MOLECULAR PHYLOGENY OF ZINGIBEROIDEAE

A Thesis
Submitted in Partial
Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

By

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Dedicated
To
My Ma and Baba

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOTECHNOLOGY

STATEMENT

I hereby declare that the matter embodied in this thesis entitled "Molecular phylogeny of Zingiberoideae" is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, India under the joint supervision of Dr. Latha Rangan and Dr. Vikash Kumar Dubey.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work of other investigators are referred.

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CERTIFICATE

It is certified that the work described in this thesis entitled "**Molecular phylogeny of Zingiberoideae"**, submitted to Indian Institute of Technology Guwahati, India for the award of degree of Doctor of Philosophy, is an authentic record of results obtained from the research work carried out under our supervision at the Department of Biotechnology, Indian Institute of Technology Guwahati, India and this work has not been submitted elsewhere for a degree.

Dr .Latha Rangan
Associate Professor
(Thesis Supervisor)

Dr. Vikash Kumar Dubey
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ABSTRACT

The investigation corroborates generation of molecular tools for understanding the molecular phylogenetics of the sub family Zingiberoideae. Results presented include proper systemic characterization according to the ethno-medicinal significance, chromosome number and associated karyo morphometric analysis, genetic assessment at the inter-varietal level, inter species and inter generic level for the genotypes, development of flow cytometric protocol for estimating nuclear DNA amounts in absolute units and understanding the dynamics of chromosome number, nuclear DNA content and development of genome size based phylogeny.

An important aspect for a proper phylogenetic assessment is understating the ethno medicinal significance. The field study during the collection of germplasm was also supported by the questionnaires of the herbal healers of the locality based on their use in traditional medicine followed by documentation. The survey revealed that the rhizome is the main part which is being used for herbal medicinal preparation and for treating gastrointestinal problems.

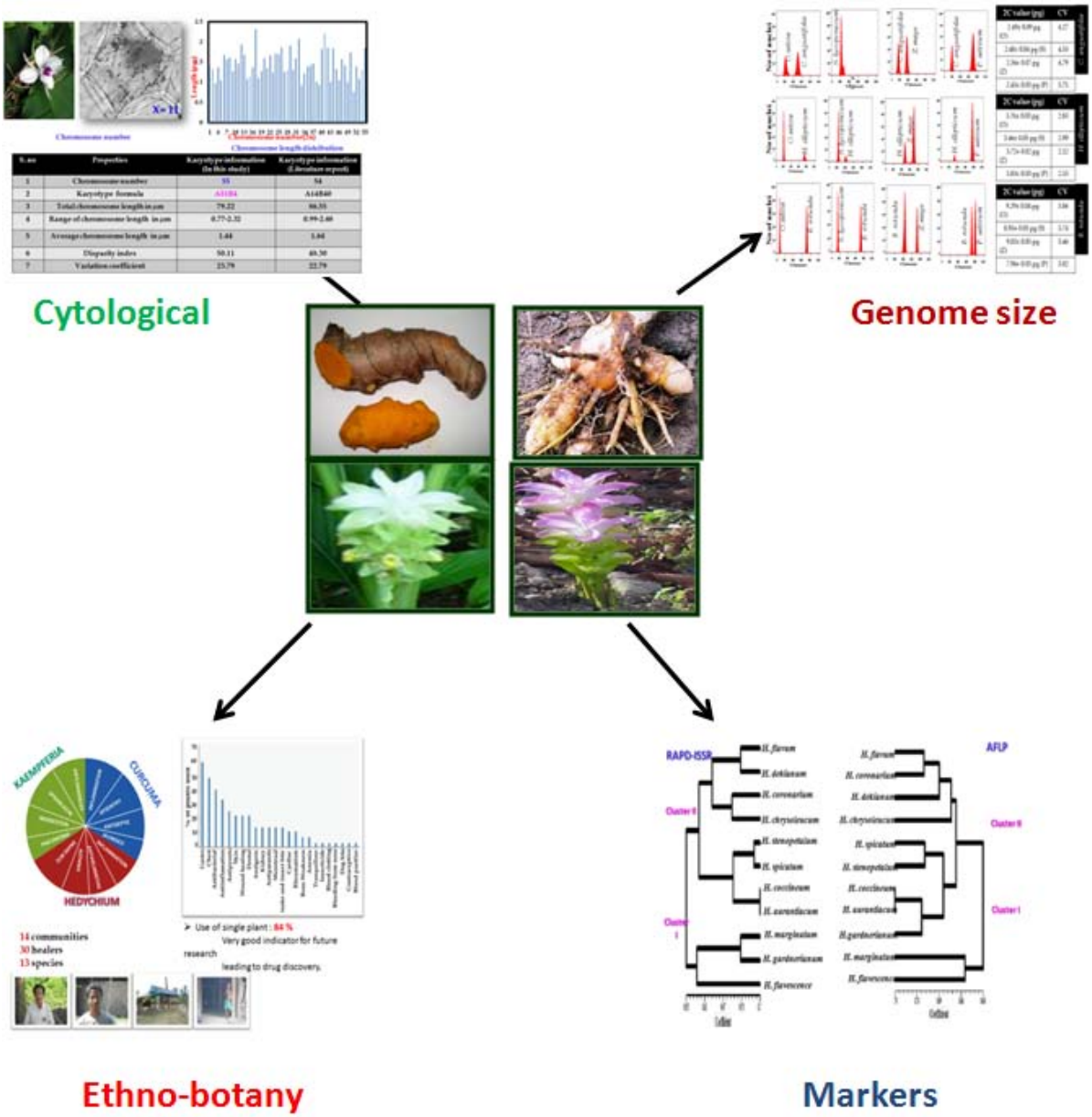
The protocol of rapid squash technique was optimised for determination of somatic chromosome count. The chromosome numbers were investigated for 9 species ranging from diploid to nonploid. The chromosome count for two species was reported for the first time. The comparatively high disparity index (DI) value found in *H. chryoleucum, C. zedoaria* and *K. galanga* corresponds to the heterogeneous assemblage of chromosomes in these species. While lower values of DI found in other members point (*C. caesia, C. longa, H. coronarium, H. gardnerianum, K. angustifolia* and *K. pulchra)* towards the general homogeneity found in various species of sub family Zingiberoideae.

Genetic assessment of the local cultivars of North East (NE) India signifies high variation implicating that turmeric genetic resources need conservation. Inter species genetic relationship showed wide variation in the genetic diversity in the wild genotypes of *Hedychium* and *Kaempferia.* The inter generic study revealed the unique genetic clustering where the species were clustered according to their genera.

The flow cytometric estimation of nuclear DNA content for the sub family Zingiberoideae was optimised in a modified nuclear isolation buffer. Statistically significant variation in nuclear DNA amounts were observed among different tissue type of six different Zingiberaceous plant studied. Intra-species variation in nuclear DNA content was estimated for cultivated turmeric (*C. longa*) variety. Population of Assam showed the highest intra-species variation of nuclear DNA content. Two fold intra-species variation was observed among the 19 cultivated turmeric varieties. Nuclear DNA content was also estimated for 26 Zingiberaceous species of which 10 were reported for the first time. Nuclear DNA content of 10 Zingiberaceous species was reported for the first time. Fold variation of 4.74 fold was reported within a narrow but bio-geographical hotspot of India. A phylogenetic tree was constructed based on the inter species variation in the nuclear DNA content.

In a sample of Zingiberaceous species, nuclear DNA content (1C) was a function of the number of chromosome compliments (2n), as represented by the linear equation $y= 18.86 x + 1052$, $r^2= 0.087$, the near zero regression coefficient signifies the chromosome number changes are due to fission and fusion with no change in DNA content. The positive correlation (r= 0.16) was observed among the dataset of chromosome number (2n) and nuclear DNA amounts (2C in pg) of the 9 studied Zingiberoideae species. A genome based phylogeny was established from the data obtained from the all the chapters which will unfold a lot of unanswered questions in the basic scientific discipline for the study of sub family Zingiberoideae.

GRAPHICAL ABSTRACT



SYNOPSIS

The use of herbal medicine is growing at a rate of approximately 20% every year (Winslow & Knoll 1998). This growing popularity has led pharmaceutical and drug development companies to capitalize on the interest of the population by searching for new herbal remedies to be used as natural products or as pharmaceuticals. Currently the benefits of herbal medicine are under intense debate in the biomedical community. An interdisciplinary approach integrating genomics and traditional medicine is therefore worth exploring as a search for phenotypes that may collectively be considered for genotyping.

The research work reported in this thesis involves understanding of ethnobotanical, karyological, genetic relationship analysis and nuclear DNA content estimation of sub family Zingiberoideae of North East India with the following objectives:

- to prepare ethnobotanical survey and the chromosome atlas of the sub-family,
- assessments of genetic relationship at inter-varietal, inter-species and inter-generic level and
- to find out the genome content using flow cytometry and the dynamics of chromosome number and genome size variation.

The work has been designed on the basis of five different chapters. Results of the present investigation are presented in three chapters (3-5). These chapters are preceded by **Chapter one** containing an introduction which provides the basic information of the work. **Chapter two** gives literature review stressing the usage of Zingiberoideae in traditional medicine, cytological studies, marker studies and nuclear DNA content estimation implying their usage for future studies.

Chapter three details out the collection of germplasm of Zingiberoideae, occurring wildly and also cultivated varieties in different parts of North East India. It also enumerate about the systematic identification of germplasm by morphological characters. The ethno-pharmacological information was collected from the herbal healers of the ethnic communities of the sub family Zingiberoideae. Cytological studies of selected species of the sub-family Zingiberoideae were carried out too.

Chapter four depicted the assessment of genetic diversity and relationship assessment of *Curcuma*, *Hedychium* and *Kaemferia*. Chapter IVA contains genetic diversity assessment of the cultivated turmeric varieties of Northeast India. Chapter IVB describes genetic relationship of *Hedychium*. Chapter IVC contains genetic relationship of *Kaempferia* and finally chapter IVD describes the inter-generic relationship for Zingiberoideae.

Chapter five presents flow cytometric estimation of Zingiberoideae species. Chapter VA deals with the optimization of the plant flow cytometric analysis. Chapter VB describes the determination of optimum tissue types for flow cytometric study. Chapter VC describes the intra species variation in nuclear DNA content of turmeric varieties of North East India. Chapter VD depicts estimation of nuclear DNA content of Zingiberoideae species. Chapter VE gives the dynamics of chromosome number and nuclear DNA content variation study.

The work described in the thesis and the related research carried out during the period of doctoral thesis has been peer-reviewed and resulted in the following publications.

Published articles:

Tushar*, **S Basak***, GC Sarma, L Rangan (2010). Ethnomedical uses of Zingiberaceous plants of Northeast India. Journal of Ethnopharmacology 132(1):286-96 (* denotes equal contribution).

Aadi Moolam Ramesh*****, **Supriyo Basak***, Rimjhim Roy Chowdhury, Latha Rangan (2013). Flow cytometric estimation of nuclear DNA content of *Pongamia pinnata* L. ***Applied Biochemistry and Biotechnology***. DOI 10.1007/s12010-013-0553-z.

Manuscript under review:

Supriyo Basak, Aadi Moolam Ramesh, Sudip Mitra, Ajay Parida, Latha Rangan (2013). Molecular phylogeny of *Hedychium* from Northeast India as dissected using PCA analysis and hierarchical clustering. ***Plant Biosystems***. (Submitted).

Supriyo Basak, Latha Rangan (2013) Chromosome number, Flow cytometric estimation of nuclear DNA content, phylogenetic and microscopic analysis of Zingiberaceae of North east India. ***Plos One*** (Submitted)

Conferences:

Supriyo Basak and Latha Rangan (2012) Genome size estimation of some selected species of Zingiberaceae from North East India. 6th International symposium on the family Zingiberaceae Department of Botany, University of Calicut, Kerala- 673635, India pp-24.

Supriyo Basak, Tushar, V. Kesari, Archana Das, Vikash Kumar Dubey, and Latha Rangan(2010). Phylogenetic analysis in Zingiberaceae native to northeast India using RAPD markers International Conference on Genomic Sciences- Recent Trends (ICGS-2010) School of Biological Sciences, Madurai Kamraj University, Madurai-625 021. pp-120.

A B B R E V I A T I O N S

AAD	Arbitrarily amplified dominant marker
ACL	Average chromosome length
AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of molecular variance
ANOVA	Analysis of variance
DI	Disparity index
GD	Genetic diversity
GDP	Gross Domestic Product
GUBH	Gauhati university botanical herberium
h	Nei's gene diversity
HCA	Hierarchical clustering
HCl	Hydrogen chloride
I	Shannon's information index
IPNI	International Plant Name Index
ISSR	Inter-simple sequence repeat
MBN	1-bromonapthalene
MI	Marker index
Ne	Number of alleles
NPB	Number of polymorphic bands
NTSYS	Numerical taxonomy system
OQ	8-hydroxyquinolene
PCA	Principal component analysis

PCR	polymerase chain reaction
PDB	Para dichloro benzene
PGR	Plant genetic resources
PI	Propidium iodide
RAPD	Random amplified polymorphic DNA
SSR	Simple sequence repeat
TCL	Total chromosome length
TNB	Total number of bands
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
VC	Variation coefficient

UNITS

bp	Base pair
^{0}C	degree celsius
Gbp	Giga base pair
g	gram
Mbp	Mega base pair
µg	microgram
µg/µl	microgram per microlitre
µg/ml	microgram per milliliter
µl	microlitre
µm	micrometer
µM	micromolar
mg	milligram
mg/ml	milligram per millilitre
ml	milliliter
mM	millimolar
min	minute
pH	negative log H^{+} ion
pg	picogram
s	second
v/v	volume/volume
w/v	weight/volume

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CHAPTER 1

INTRODUCTION

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This chapter gives an introductory note about ethno-botany, cytology, molecular markers and genome size and finally the scope and objectives of the thesis work.

INTRODUCTION

1.1 Background

The three basic needs of human existence food, clothing and shelter are provided by plants. Although, plant diversity has been characterised to be 3, 00,000 species of higher plants, only about 7,000 (2.3 %) species have been domesticated for food, fodder and feed, of which 30 (0.01 %) species deliver the dietary energy for human survival. While the number of cultivated species for human survival is so small, high level of intra species, inter species and inter generic diversity in crop plants and their wild relatives leads to the development of plant genetic resources (PGR). International Undertaking on Plant Genetic Resources defines PGR as the reproductive or vegetative propagating material of (i) cultivated varieties (cultivars) in current use and newly developed varieties; (ii) obsolete cultivars; (iii) primitive cultivars (landraces); (iv) wild and weed species, near relatives of cultivated varieties; and (v) special genetic stocks (including elite and current breeder's lines and mutants) (FAO 1983). PGR, according to the Convention for Biological Diversity, are any living material of present and potential value for humans (CBD 1992). Plant genetic resources consisted of all agricultural crops and some of their wild species because they possess valuable traits. Plant genetic diversity is susceptible to “genetic erosion”, the loss of individual alleles/genes and of combinations of alleles/genes, such as those found in locally adapted landraces. Genetic erosion is caused by the replacement of local varieties by modern varieties which are not compatible in resisting them with the disease, pest attack accompanied by climate change, environmental degradation and urbanization. Plant genetic resources being the storehouse of genetic diversity will face the challenge of crop adaptations to different biotic and abiotic stresses by providing valuable traits. According to FAO, replacement of local varieties by modern varieties resulting in reduction of the sheer number of cultivars is the main cause of genetic erosion. This is intensified by the emergence of new pests, weeds and diseases, environmental degradation, urbanization and land clearing.PGR, the

only source of plant genetic diversity, provides valuable traits needed for meeting the challenges of adapting crop varieties. An individual genotype with seemingly useless set of characters today may suddenly become essential tomorrow due to changing climatic conditions or outbreaks of disease. For example, *Zea diploperennis*, a wild relative of the cultivated species of corn (*Zea mays*) has saved corn cultivation in West Africa and increased its production many folds (Lane et al1997, Eubanks & Cook 1999). The contribution of the wild Indian rice species *Oryza nivara* to the development of new varieties resistant to grassy stunt virus is another concrete example of usage of PGR in the crisis.

Therefore, it has been long realized to conserve the diversity that exists. The modern intensive agriculture calls for uniformity and consequently has a narrow genetic base. In contrast, traditional agriculture has large number of diverse landraces. To conserve the diversity found within species of cultivated plants, experts employ a strategy that combines *ex situ* conservation (storing diversity in gene banks) with *in situ* on-farm conservation in matching agro-ecosystems.Every country has the responsibility to conserve, restore and sustainably use the plant genetic resources within its jurisdiction. India, a mega biodiversity country, while following the path of development, has been sensitive to needs of conservation and is still rich in plant genetic resources. In terms of plant diversity, India ranks fourth in Asia and tenth in the world (Sharma & Goel 2007).

India's contribution to agro-diversity has been impressive. It hoses 47,000 species of flowering and non-flowering plants which represents 12% of the recorded world's flora. The products derived from natural plant bio resources have contributed significantly to the Indian GDP (Gross Domestic Product) for example self-sufficiency in food, reduced import of edible oil, and export of high yielding horticultural products like tea, turmeric, pulses and timber. India hoses two out of 18 biodiversity hotspot of the world namely Western Ghats and Eastern Himalayas. Western Ghats, the richest centre of endemism in India encompasses the states of Gujarat and Maharashtra, Goa, Karnataka, Tamil Nadu and Kerala. Eastern Himalayas comprised of Nepal, Bhutan and neighbouring states of East, a contiguous sector Yunnan province in South Western China and North East (NE) India.

The NE region (located between 87°32'E to 97°52'E longitudes and 21°34' N to 29°50'N latitude) known for its genetic resources world over, is the Bio-geographical Gateway to India's richest biodiversity zones and finds place in part of two-biodiversity hotspots in India (Mittermeier et al 2003). NE politically consists of Arunachal Pradesh (AP), Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura. The unique richness of bio-resources in NE makes it a potential hub for economic growth of the country to a competent level if utilized and tapped efficiently. The region is rich in medicinal plants and many other rare and endangered taxa.

There is a huge patronage of herbal products around the world as an alternative to orthodox drugs and these medicinal plants have immensely contributed to the development of human health and welfare (Lee & Xiao 2005).Ethno-medicine may be defined broadly as the use of plants by humans as medicines (Farnsworth et al 1990, 1994); but this use could be called more accurately ethno botanic medicine. Ethnopharmacology is a highly diversified approach to drug discovery involving the observation, description, and experimental investigation of indigenous drugs and their biologic activities. It is based on botany, chemistry, biochemistry, pharmacology, and many other disciplines (anthropology, archaeology, history, and linguistics) that contribute to the discovery of natural products with biologic activity (Rivier &Bruhn1979). Medicinal plants are the biggest source of herbal medicine amongst which Zingiberaceae is of utmost significance. Curcumin, an active compound isolated from Zingiberaceous plant, has wide range of medicinal efficacies (Nagpal & Sood 2013).

1.2 Zingiberaceae

Zingiberaceae are one of the eight families of the order Zingiberales in monocotyledons. Zingiberaceae occurs chiefly in the tropics consisting of 52 genera and harbouring about 1500 species (Sirirugsa 1999). India is one of the richest regions for its diversity, having 22 genera and about 170 species. The NE India is a zone of greatest concentration where 19 genera and about 88 species are reported (Prakash & Mehrotra 1996).

Based on vegetative and floral characteristics, the presently established cataloging of the Zingiberaceae (Petersen 1889; Schumann 1904; Holttum 1950;

Burtt and Smith 1972; Smith 1980; Larsen and Mood 1998) divides the family into four tribes (Hedychieae, Alpinieae, Zingibereae and Globbeae). On the basis of molecular data, a realignment of the genera of the Zingiberaceae into four subfamilies (Kress et al 2002): the Siphonochiloideae (the genus Siphonochilus only), the Tamijioideae (the single genus Tamijia), the Alpinioideae (most of the former Alpinieae), and the Zingiberoideae (including the former tribes Hedychieae, Zingibereae and Globbeae) was done (Fig 1.1).The sub family Zingiberoideae consists of medicinally important genera of *Boesenbergia, Caulokaempferia, Cautleya,Curcuma,Curcumorpha, Haniffia, Hedychium, Kaempferia, Scaphochlamys* and *Stahlianthus. Curcuma, Kaemferia* and *Hedychium* are taken as the representative genera under this thesis purview as these genera are available widely throughout the NE India and the taxonomic revision is neglected due to their inaccessible natural habitat and difficulties in collection from dense tropical forests. For many genus of sub family Zingiberoideae, our knowledge is still insufficient, even about the basic morphological characters often making the proper discrimination difficult. In addition, high intra and inter population variation has led to debate concerning species concepts and boundaries. As a result, one species has often been described repeatedly under different names whereas the same name has been applied to different taxonomic entities.

1.2.1 Genus: Curcuma

The genus *Curcuma* L. is composed of about 70-80 species of rhizomatous annual or perennial herbs (Purseglove 1974; Sirigusa 1999). It has paramount significance as spice, medicines, dyes, cosmetics, starch and ornamentals. *C. longa* is one of the important members of the family. It yields turmeric which is used as colouring ingredient of Indian cuisine (Purseglove, 1974; Aravatjrut et al 1999). Turmeric is also used in pharmaceutical industry due to its medicinal significance (Majeed et al 1995). The finest Indian arrowroot is derived from *C. angustifolia* (Das et al 1999). The species belonging to the genus *Curcuma* can be grown in diverse tropical conditions from sea level to a height of 1500 m on the hilly slopes, in the temperature ranging from 20-30 °C. produce seeds. They reproduce asexually by means of rhizomes.

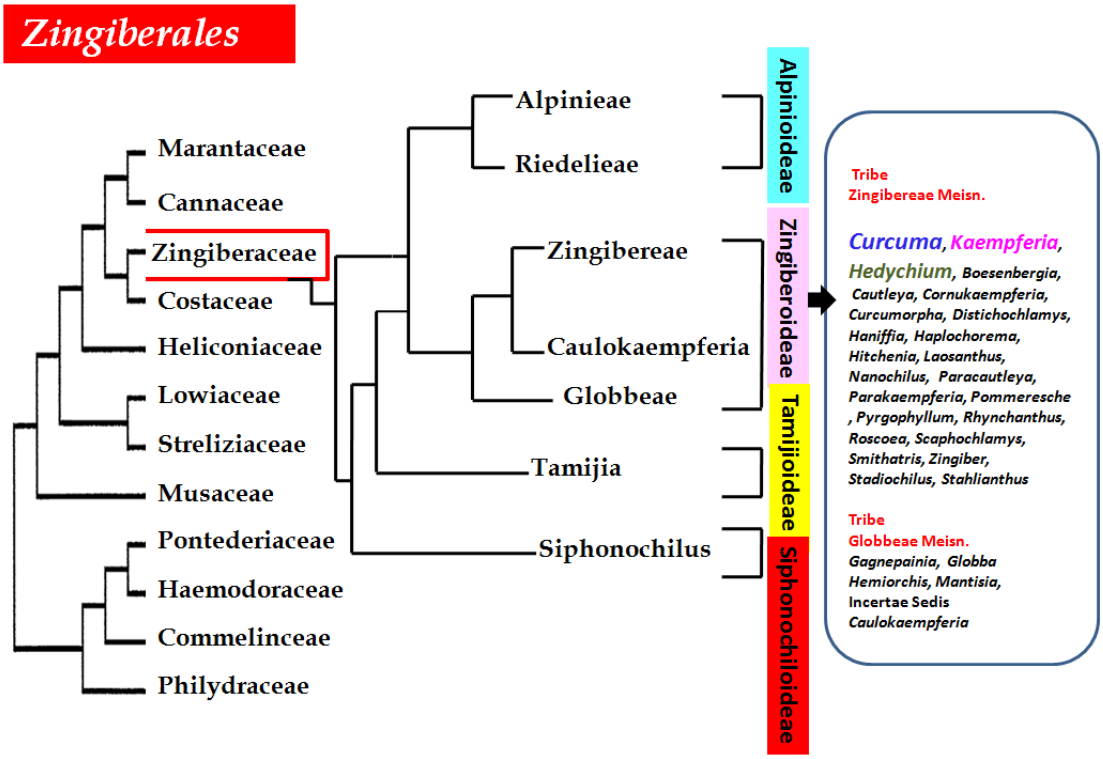

Fig 1.1 Cladogram showing the system of the Zingiberaceae (Kress et al 2002)

The genus can easily be recognized by its large compound spike inflorescence bearing prominent spiral bracts, which laterally fuses to form pouches. *Curcuma* species do not

1.2.2 Genus: Hedychium

Hedychium J. König is an economically important genus, consisting of 65 species worldwide of which NE India has the highest species concentration (24 out of 65 Wood et al 2000; Hamidou et al 2008). *Hedychium* species are widely cultivated for their perfume, and as a useful raw material for manufacturing paper. Moreover, some species are cultivated for their edible flowers (He 2000). The genus is also of horticultural importance. The inflorescence of *H. coronarium*, the national flower of Cuba, is aromatic and showy, resembling a cluster of flying white butterflies. Flowering in *Hedychium* mainly occurs in summer and autumn, while a few species bloom in winter and spring.

1.2.3 Genus: Kaempferia

The genus *Kaempferia* L. comprises about 60 species geographically distributed from India to Southeast Asia (Sirirugsa 1992; Larsen & Larsen 2006), with a concentration of 22 species from British India (Baker 1890). One of the important species of the genus is *K. galanga* (sand ginger) used as a herb in cooking in parts of Indonesia and Thailand. Taxonomic classification of *Kaempferia* is tricky on account of the morphological similarity of vegetative parts amongst the species of the same genus and other genera in Zingiberaceae, such as *Boesenbergia*, *Cornukaempferia*, *Curcuma*, and *Scaphochlamys*, while the taxonomic identification based on reproductive parts is limited due to short flowering season. In NE India, most *Kaempferia* species are dormant during November to early May. Therefore, a combination of several diagnostic characteristics, both vegetative and floral morphology, is essential for taxonomic delimitation at any species level.

1.3 Chromosome studies

Cytology plays an important role in taxonomy to achieve a classification which represents mutual relationships and is useful in indexing plants. Modern taxonomy

takes up cytological evidence based on chromosome numbers, chromosome morphology, and chromosome behaviour in meiosis and in aberrant forms of reproduction. Karyotype analysis has been proved to be very effective for assessing taxonomic relationships in many cases (Gao et al 2012). Comparative karyotype analysis of closely related species has been performed in many cases to explain patterns and directions of chromosomal evolution and to deduce the evolutionary role of karyotype changes (Shan et al 2003)

Chromosome number is necessary to elucidate the structure, function and organization of sub family Zingiberoideae plants genes and genomes. However, few researches about chromosome observation of Zingiberoideae were done, not only because their chromosome numbers are large, but also because their ploidies are complicated. Also, Zingiberoideaeare characterized by small chromosomes that are difficult to karyotype.

1.4 Genetic diversity

Genetic diversity has been defined as the variety of alleles and genotypes present in a population and this is reflected in morphological, physiological and behavioural differences between individuals and populations (Frankham et al 2002). Gene and chromosome mutation in individuals and recombination through sexual reproduction are the prime causes of new genetic variation. The structure, number of chromosome and the amount of DNA per cell are other classes of genetic diversity. Genetic variation results in natural evolutionary change and artificial selective breeding (Thomas 1992). From a descriptive point of view the genetic information can refer to individual genes, DNA sequence, chromosomes or quantitative genetic variation. Molecular markers (RAPD, ISSR and AFLP) have been used in the characterisation of genetic diversity and genetic diversity has been estimated by four parameters viz; 1) frequency of genotypes and alleles 2) percentage of polymorphic loci 3) observed and expected heterozygosity 4) allelic diversity.

The knowledge of genetic variability is a pre requisite to study the evolutionary history of a species, as well as for other studies like intra specific variations, genetic resources conservation etc (Islam et al 2007). The biodiversity study unfolds the quantum of variation prevailing in a given species per unit area,

which help to devise appropriate conservation study for future (Mandal et al 2007). Genetic diversity (GD) is sometimes considered to be the invisible dimension of biological diversity. The present structure of GD is the result of the evolutionary history of the species exposed to natural selection pressures in variable environmental conditions. A species that has a large degree of genetic diversity among its population will have more variations from which to choose the fit alleles. Increase in GD is also essential for an organism to evolve. Species that have very little genetic variation are at a great risk. The vulnerability of a population to certain types of diseases can also increase with reduction in GD. Genetic variation in crop plants has continued to narrowed down due to continuous selection pressure for specific traits i.e. yield, thus rendering them more vulnerable to disease and insect epidemics and jeopardizing the potential for sustained genetic improvement over a long term (Harlen 1987). However, members of Zingiberaceae mostly being vegetatively propagated plants face less natural selection pressure; still they are facing the loss of GD, due to uncontrolled uprooting of the rhizome and the whole plant in huge quantities for preparation of herbal medicine. This indiscriminate collection of rhizomes coupled with vanishing forests and grasslands resulted in the depletion of Zingiberaceae in the wild. Many of the Zingiberaceous plants are found throughout the NE India in the wild state. The members of this family are mostly neglected due to their inaccessible natural habitat and difficulties in collection from dense tropical forests. Unless some strong conservation measures are taken, many members will move to extinct category in the near future. Studying genetic diversity using molecular markers would therefore be useful for any kind of future analysis using the members of the family Zingiberaceae. Also the molecular systematic approach will be significant especially towards protection and utilization of bioresources from NE India (Rangan et al 2008).

1.5 Genome size

Most of the hereditary materials are carried by the plant cell nucleus. Estimation of nuclear DNA content [C-value (Greilhuber et al 2005) in picograms and number of base pairs] has led to the discovery of 2000 fold variation in the genome size of plants, with the smallest known genome 63 Mbp (Greilhuber et al 2006) found in *Genlisea margaretae* (Lentibulariaceae) and the largest genome of 127 Gbp in the

tetraploid *Fritillaria assyriaca* (Liliaceae) (Leitch et al 2005). The availability of data on genome size is critical for many fields of research, including taxonomy and evolutionary changes (Kron et al 2007). Its knowledge is essential for planning gene cloning and genome sequencing projects (Rabinowicz& Bennetzen 2006). The relationship between ploidy and nuclear DNA content makes the assay suitable for the determination of ploidy level, detection of mixoploidy and, under certain conditions, also aneuploidy (Dolezel et al 2007). The applications range from taxonomy and population biology to breeding and quality control in seed production (Suda et al 2007). Simultaneous estimation of DNA content in seed embryo and endosperm makes it possible to determine the genetic origin of a seed, that is, establish its sexual or apomictic origin and gametic ploidy (Matzk 2007; Barow &Meister 2003). More specialized applications include the presence and extent of endopolyploidy (Barow & Meister 2003) and the estimation of DNA base content (Meister & Barow 2007).

A complete understanding of genomes is still data poor since insufficient number of species examined. Recent estimates indicate that genome size has been estimated for roughly 1.8% of angiosperms (Bennett & Leitch 2005). In 2005, a special issue of *Annals of Botany* on plant genome size acknowledged the need for improved representation of the global flora (Gregory et al 2007).

Many of the current questions regarding genome size variation will require additional data from a large taxonomic distribution. A variety of methods have been used to estimate genome size: Feulgen microdensitometry, reassociation kinetics, fluorometry, biochemical analysis, and more recently FCM. The most widely used method has been microdensitometry; the use of FCM has markedly increased, at least in plants (Bennett & Leitch 2005). However data is lacking in the context of nuclear DNA content of NE Indian Zingiberoideae plants. Present study aims to provide comprehensive information about the nuclear DNA content estimation of some of the members of Zingiberoideae plants. This knowledge will provide valuable information for gene cloning and sequencing studies.

1.6 Specific objectives

1. Collection of germplasm occurring wildly and also cultivated variety in different parts of North East India.
2. Systematic identification of collected germplasm using various keys specifically morphological traits.
3. Ethnobotanical survey of the sub family Zingiberoideae (*Curcuma*, *Kaempferia* and *Hedychium*) in Northeast India.
4. Chromosome number observations and karyo-morphometric analysis of selected species of the sub family Zingiberoideae.
5. Genetic assessment at the inter-varietal level of turmeric (*C. longa*).
6. Genetic relationship study of wild germplasm of *Hedychium* and *Kaempferia* at the inter species level.
7. Optimisation of flow cytometric protocol for estimating nuclear DNA content in absolute units.
8. Variation of nuclear DNA content in different tissue types.
9. Estimation of intra species variation of nuclear DNA content of turmeric variety of NE India.
10. Nuclear DNA content estimation of Zingiberoideae.
11. Dynamics of chromosome number and nuclear DNA content.

1.7 Scope and significance

1. Systematic collection and maintenance of *Curcuma, Kaempferia and Hedychium*, the three promising genera of the sub family Zingiberoideae from NE India will throw immense insights towards pharmacological, economic and environmental significance.
2. Detailed ethanobotanical study of the sub family Zingiberoideae will document the hidden medicinal prescription of the traditional communities found in species occurring in different parts of NE India. This will provide basic information for the discovery of novel lead compounds from the Zingiberoideae plants.
3. Chromosome studies of some of the important members of sub family Zingiberoideae will provide the necessary information of their chromosome number (2n), basic chromosome number (x), polyploidy status, karyomorphometric analysis and cytological evolution.
4. Genetic diversity assessment of the local cultivars of NE India will shed light towards the conservation of turmeric genetic resources.
5. Inter species genetic relationship of wild genotypes of *Hedychium* and *Kaempferia* will indicate the amount of genetic diversity present in the germplasm.
6. The flow cytometric estimation of nuclear DNA content for the sub family Zingiberoideae will open the door for further molecular studies.
7. Nuclear DNA content variation in different plant part will question the consistency of nuclear DNA amounts in a particular species, if any.
8. Intra-species variation in nuclear DNA content of cultivated turmeric (*C. longa*) variety will depict nuclear DNA content in a population of single species and finally.
9. The fold variation persisted in the different species of Zingiberoideae will point towards the variable amounts of repeat elements.

CHAPTER 2

LITERATURE REVIEW

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This chapter gives the description of subfamily Zingiberoideae with the key highlights of the each of the techniques used. The chapter also provides the details of the previous work carried out.

LITERATURE REVIEW

2.1 Zingiberaceae: An overview

Family Zingiberaceae belonging to Monocotyledons consists of 53 genera and over 1200 species (Kress 1990). It harbors perennial, attractive aromatic plants yielding spices, condiments, dyes, perfumes and medicines besides many ornamental species cultivated for their showy flowers (Borah et al 2012) (Fig 2.1). It is chiefly present in tropical regions confined mainly to Asia, Africa, tropical America and Australia, Indo - Malaya region being the main centre of its occurrence (Burtt and Smith, 1972, Jain and Prakash, 1995). Species of the Zingiberaceae are the terrestial plants mostly growing in damp and humid shady places. They are also found in topical forest. Some species can fully expose to the sun, and grow on high elevation (Sirirugsa 1990). The first attempt on detailed study of Zingiberaceae in 20th century was made by Schumann (1904) who discussed almost all the available species of that time with detailed description, correct nomenclature including all available synonyms and key to the species and genera. Later Holttum (1950) published a detailed account of the Zingiberaceae of Malay Peninsula. The other notable contributions on Zingiberaceae are those of Burtt (1972), Burtt and Smith (1972a and b, 1983). In India, the earliest notable contribution was that of Roxburgh (1812) who reported 8 genera and 47 species from India and added 19 species in his Flora Indica in 1832. Baker (1890-92) presented an account of Zingiberaceae in Hooker's Flora of British India. However, there are different estimates of number of genera and species by different workers. Karthikeyan and Mudgal (1995) in their article in "Bharat ki Vanaspatik Vividhata" listed 22 genera and 167 species of Zingiberaceae. Again, Jain and Prakash (1995) in an article on phytogeography and endemism of Zingiberaceae reported 22 genera and 178 species from India distributed in Eastern Himalaya, NE India, Western Ghats and Andaman and Nicobar islands and only a few showing representation in Central India and Western Himalaya.

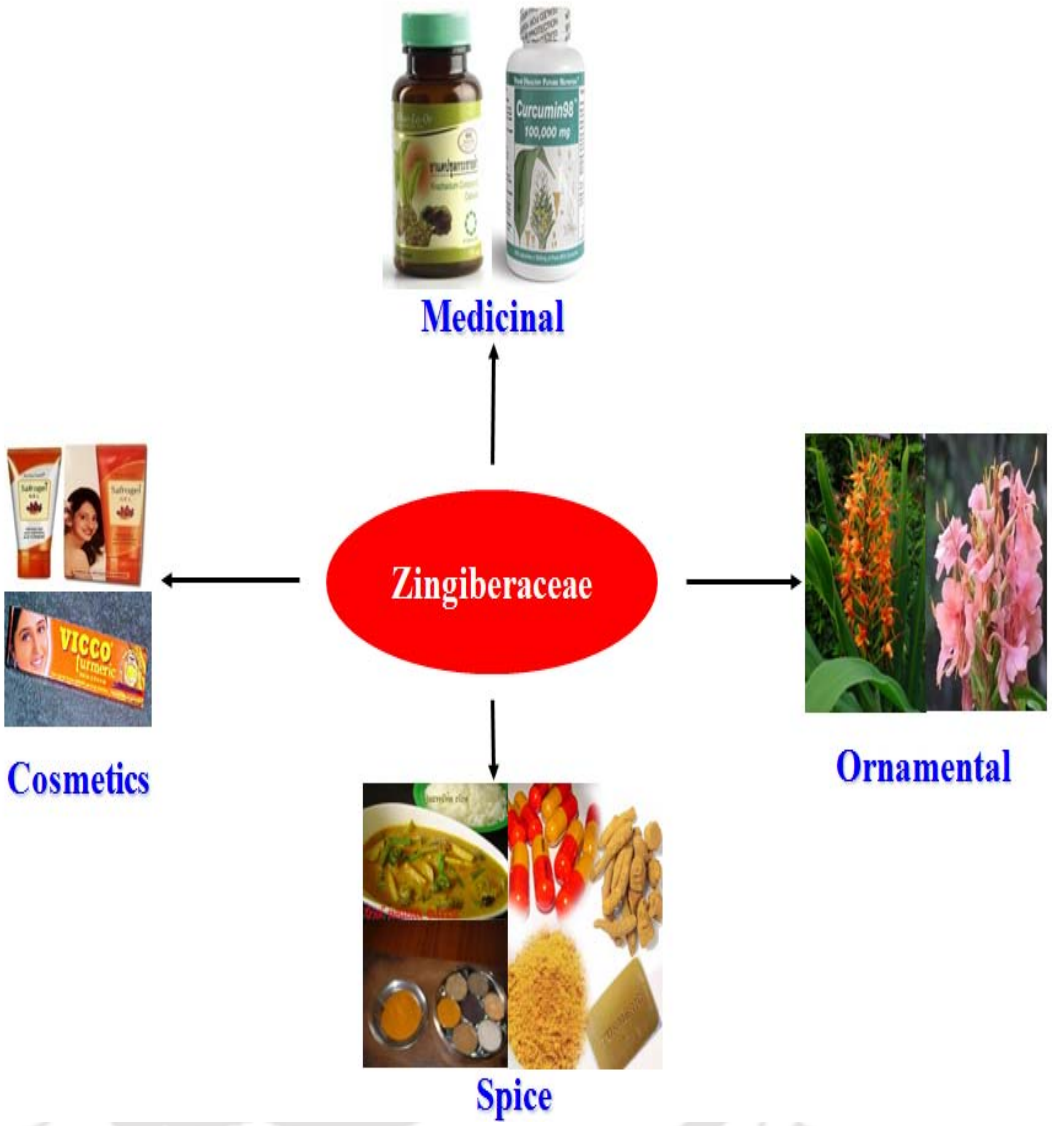

Fig 2.1 Significance of Zingiberaceae

Classifications of the family, first proposed in 1889 and refined by others since that time, recognize four tribes (Globbeae, Hedychieae, Alpinieae, and Zingibereae) based on morphological features (Holttum 1950) (Fig 2.2). Most of the characters that are used to define the tribes are inconsistent and variable. A consensus has not been reached on the actual number of genera in the family. New phylogenetic analyses have been used to propose a new classification of the Zingiberaceae that recognizes four subfamilies and six tribes: Siphonochiloideae (Siphonochileae), Tamijioideae (Tamijieae), Alpinioideae (Alpinieae, Riedelieae), and Zingiberoideae (Zingibereae, Globbeae) (Kress et al 2002).

Siphonochiloideae (Siphonochileae) consists of plants with seasonal dormancy period and also characterized by fleshy rhizomes and trilocular ovary with axial placentation. Tamijioideae (Tamijieae) has been characterised by evergreen plants with fibrous rhizomes; ovary unilocular with parietal placentation. Subfamily Alpinioideae has been characterised by the reduced or missing lateral staminodes. The key character determining Riedelieae is the presence of extra floral nectaries on leaf blades, silique-like fruits opening by longitudinal slits. Alpinieae has been categorized by the absence of extra floral nectaries, fleshy or indehiscent fruits. Subfamily Zingiberoideae has been characterized by the plane of distichy of leaves parallel to rhizome. Within the subfamily, the tribe Zingibereae has been characterised by the trilocular ovary with axial, basal or free columnar placentation. Tribe Globbeae has been characterised by the unilocular ovary with parietal placentation, labellum often connate to filament in a slender tube (Reveal 2002) (Fig 2.3).

2.*1.1 Zingiberoideae*

Subfamily Zingiberoideae contains the members of the previously placed tribes Zingibereae, Hedychieae and Globbeae. The striking floral features of this subfamily are the well-developed lateral staminodes that are generally absent in the Alpinioideae (Fig 2.4 A).

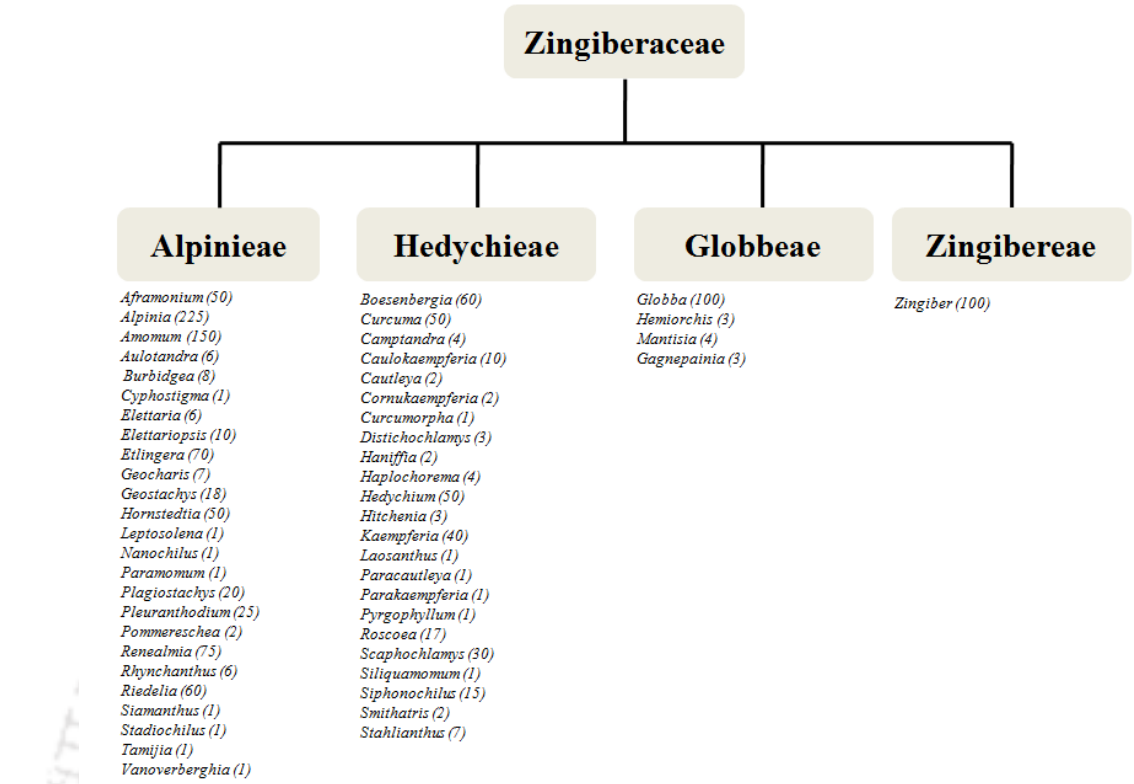

Fig 2.2 Classification of Zingiberaceae (Holtum's 1950). Number of species has been mentioned in parenthesis.

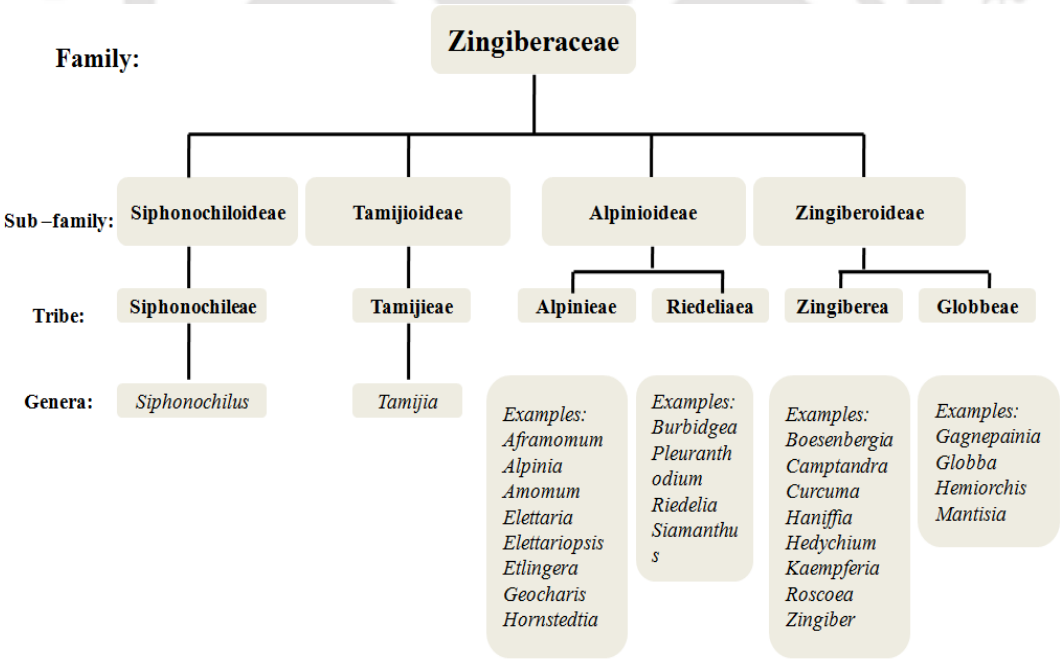

Fig 2.3 Classification of Zingiberaceae based on molecular data (Kress et al 2002).

The most prominent vegetative feature that is universal in this subfamily is the parallel orientation of the plane of distichy of the leafy shoots with respect to the rhizome (perpendicular orientation in all other Zingiberaceae) (Fig 2.4 B). A second vegetative trait that characterizes the Zingiberoideae is the capacity for members of this subfamily to go into dormancy during the dry season (Fig 2.4 C). Members of Alpinioideae cannot be forced into dormancy, possess tough fibrous rather than fleshy rhizomes, and are always evergreen in their natural habitats. Zingiberoideae has been classified in to two groups based on molecular marker data (Kress et al 2002). Zingibereae contains several genera. The *Curcuma* clade consist of *Curcuma*, *Stahlianthus*, *Hitchenia*, and *Smithatris*, which share cone-like inflorescences of few-flowered, congested bracts. *Kaempferia* clade consists of *Haniffia*, *Zingiber*, *Kaempferia*, *Distichochlamys*, *Scaphochlamys*, *Boesenbergia*, *Curcumorpha* and *Cornukaempferia*. These taxa lack the distinctive pseudostem (except for *Zingiber* and *Haniffia*) found in other members of the subfamily. The taxonomy of *Hedychium* is still unresolved. The four genera in the traditionally recognized Globbeae are united by several morphological characters such as the unilocular ovary with parietal placentation (convergent with *Tamijia*) and the long-arching filament to which the labellum and/or lateral staminodes are fused (in *Globba* and *Mantisia* only).

2.1.1.1 Genus: Curcuma

The genus *Curcuma* with about 50 species has been circumscribed by its `pouched´ inflorescence bracts and versatile, usually spurred anthers (Fig 2.5). It is a diverse polyploidy complex containing many taxa of economic, medicinal, ornamental and cultural importance, the type species of the genus, *C. longa* L. (turmeric) being the best-known example (Skornickova et al 2012).

Rhizomes of *Curcuma* are branched, fleshy and aromatic. Roots often bear conical or ellipsoidal tubers. Leaves are basal and blade is broadly lanceolate or oblong or rarely linear and narrow. The genus can be easily recognized by its large compound spike inflorescence bearing prominent spiral bracts which laterally fuse to form pouches. Each pouch subtends to a cincinnus of two to ten flowers that contain a single versatile anther.

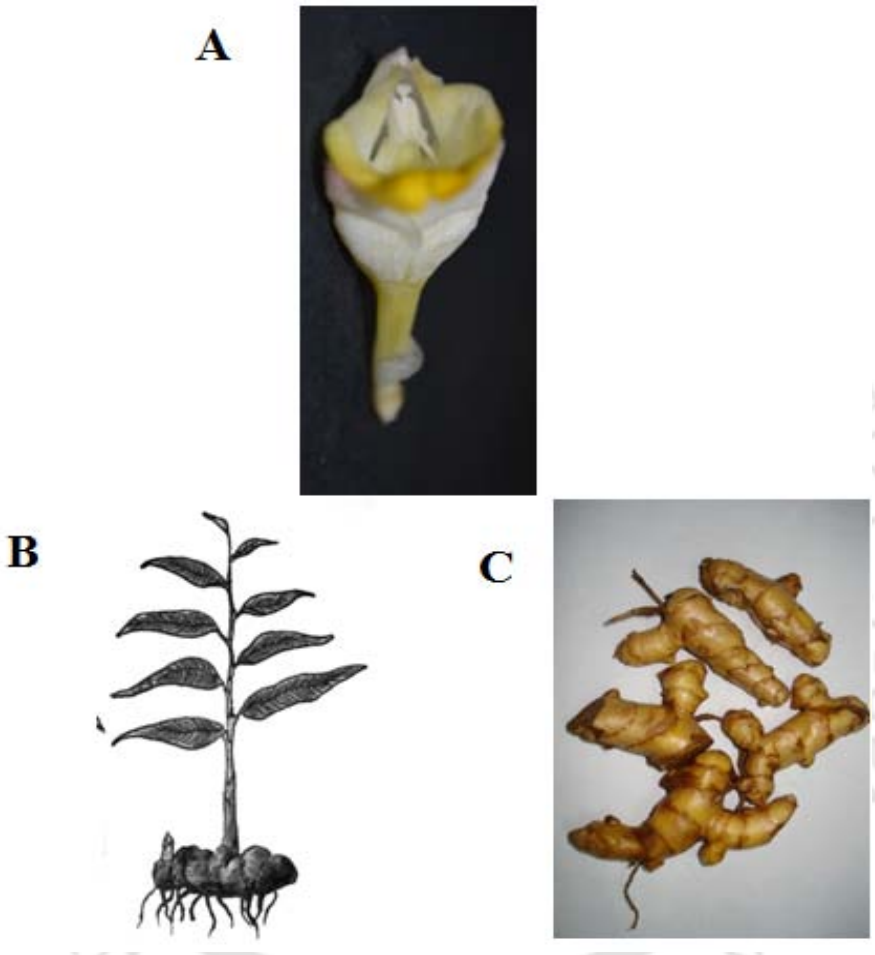

Fig. 2.4 Taxonomic features of Zingiberoideae. **A.** well-developed lateral staminodes **B.** parallel plane of distichy of leaves to rhizome (*Zingiberoideae*), and **C.** seasonal dormancy

The terminal bracts form a sterile cluster called ‘coma’ very long and often brightly colored. It has two distinct flowering times. Early flowering (April-May) species develop laterally from the rhizome before development of leafy shoot. Late flowering (August-September) species usually develop terminally from the leafy shoots (Sirirugsa 1999).

The plants vary from 50-200 cm in height. *Curcuma* species are mostly triploid and do not produce seeds. They reproduce asexually by rhizomes. The identification of the members of this genus has traditionally been achieved using morphological data. However, *Curcuma* species exhibit large morphological variations both inter and intra species, but in some cases, especially early flowering group shows a very similar pattern of morphology between them which leads to confusion in their identification (Apavatjrut et al 1999).

2.1.1.2 Genus: Kaempferia

Kaempferia comprising of more than 60 species, has distribution in tropics and subtropics of Asia and Africa. It contains many aromatic rhizomatous herbal species, which are the ingredients of many ayurvedic drug preparations, and the aromatic essential oil from the rhizome is valuable to perfumery. The vegetative characters of taxa found in Southeast Asia are often associated with the fleshy rhizomes, usually short, with several roots in a bundle. The roots of some species, particularly those with inflorescence separated from and preceding leaf shoots, are fibrous with terminal globular to fusiform storage tubers. Leaves range from one to a few; they are filiform to very broad, rising from the rhizomes, usually with keeled sheathes, short to long petioles, small, inconspicuous, or without ligules (Fig 2.6).

The inflorescence of all species found in Thailand is either separated (in all taxa preceding the appearance of the leaf-shoots), or terminal and beginning with the leaf-shoots. The terminal inflorescence often forms a pedunculate head, and all its primary bracts are always fertile. However, the radical inflorescence is either sessile or very shortly pedunculated, with 2-4 sterile sheathing bracts. The non-tubular primary bract is always accompanied by a shortly bilobed or bilobed-to-the-base bracteole, and subtends only one flower. A combination of the overall floral

morphology is characteristic of the Asiatic *Kaempferia* species. The labellum is separated from the lateral staminodes almost to the base, and is often deeply bilobed, except that of *K. parviflora* Wall. ex Baker. The lateral staminodes are always petaloid and the anther crest is always conspicuous and could be entire or dentate, straight or reflexed, and narrow or orbicular. Moreover, the stigma is always cup-shaped with ciliated rims and the stylodial glands are paired and needle-like. However, morphological features can be greatly variable, even within the same taxon. Therefore, until the range of character variation of these generic features is fully understood, a single diagnostic character cannot be used for taxonomic decision-making (Picheansoonthon & Koonterm 2008).

2.1.1.3 Genus: Hedychium

Hedychium J. König is an economically important genus, consisting of 65 species worldwide of which NE India has the highest species concentration (24 out of 65 Wood et al 2000; Hamidou et al 2008). *Hedychium* species are widely cultivated for their perfume, and as a useful raw material for manufacturing paper. Moreover, some species are cultivated for their edible flowers (He 2000). The genus is also of horticultural importance. The inflorescence of *H. coronarium*, the national flower of Cuba, is aromatic and showy, resembling a cluster of flying white butterflies. Flowering in *Hedychium* mainly occurs in summer and autumn, while a few species bloom in winter and spring. The taxonomy of the *Hedychium* genus is unclear.

Inter-specific hybrids of *Hedychium* are readily created and natural hybridisation undoubtedly contributes to taxonomic confusion (Wood et al. 2000). The taxonomy of the 'white' and 'yellow' gingers (*H. flavescens* and *H. coronarium*) is particularly confusing (Fig 2.7). For example, in New Zealand, material described as either 'white ginger' or 'yellow ginger' has been referred to as *H. flavum*, *H. flavescens*, *H. coronarium* or *H. oblongum* in various publications over the past 30 or more years.

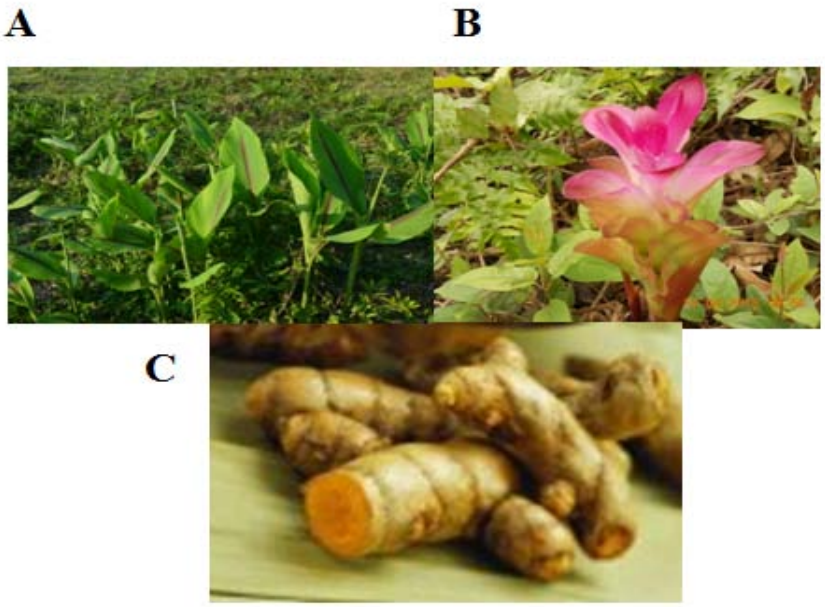

Fig 2.5 A representative *Curcuma* plant. A. Entire plant; B. Inflorescence; C. Rhizomes.

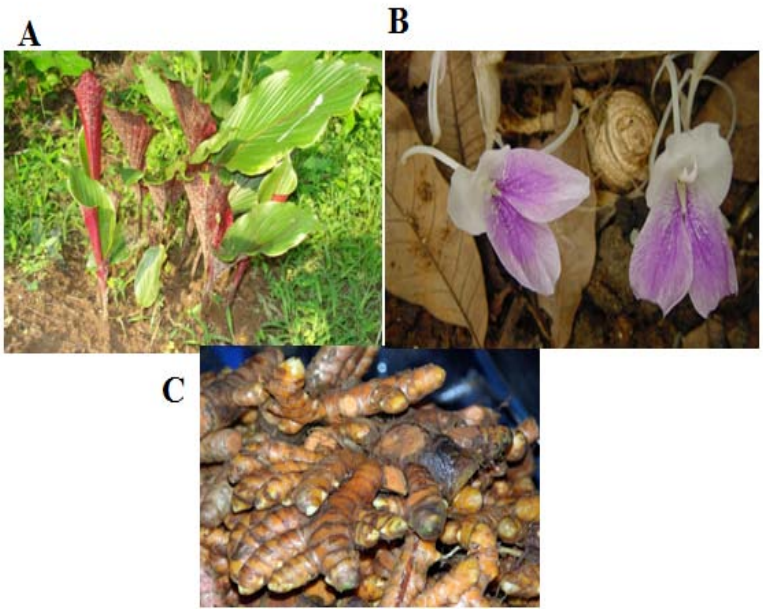

Fig 2.6 A representative *Kaempferia* plant. A. Entire plant; B. Inflorescence; C. Rhizomes

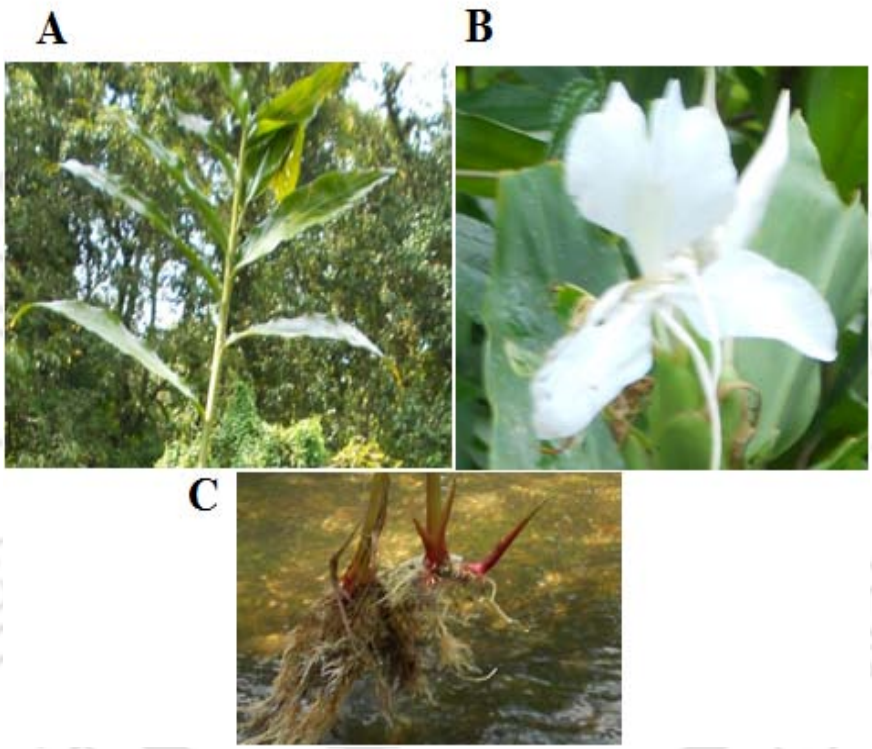

Fig 2.7 A representative *Hedychium* plant. A. Entire plant; B. Inflorescence; C. Rhizomes.

2.2 Ethnobotanical studies

Ethnobotany, as defined by Harshberger "the study of the utilitarian relationship between human beings and vegetation in their environment, including medicinal uses" (Harshberger 1896). Although the term "ethnobotany" was coined in 1895 by US botanist John William Harsherberger, the history of the field is very old. The Greek surgeon Dioscordis in AD 77 published "De Materia Medica", which was a catalogue of 6000 plants in the Mediterranean. It elucidated the information on usage of the plants for medicinal purposes. Leonhart Fuchs, a Renaissance artist, catalogued 400 plants native to Germany and Austria in 1542. John Ray provided the first definition of species in his "Historia Plantarum". Carl Linnaeus is famous for inventing the binomial nomenclature of plant. The revolution in the field of botany was seen in 19th century when Alexander von Humboldt and Captain Cook started botanical exploration.

During the past few decades a plenty of ethobotanical surveys have been carried out as the traditional uses of plants in the world have been progressively gaining considerable attention (Rivera et al 2005; De Natale & Pollio 2007). Kala et al (2005) documented the wealth of 158 medicinal plants distributed across 73 families and 124 genera used by the Apatani tribe of Arunachal Pradesh for curing 52 different types of ailments. *Asteraceae* was the most dominant family (19 species, 11 genera), followed by *Zingiberaceae, Solanaceae, Lamiaceae* and *Araceae*. The usage of the above ground parts was higher compared to the below ground plant parts in the Apatani group of the village. Saikia et al (2006) studied the ethnobotany of Assamese people for curing skin related ailments for a total of 85 plants belonging to 49 families. The usage of single or multiple plants for preparing herbal medicine and water as the medium for herbal medicine preparation was documented. Oral mode of delivery of herbal medicine was most preferred method. Saikia et al (2006) documented the cure for 18 different skin ailments, of which 14 plants were used for curing multiple skin diseases. Rout et al (2010) documented 8 plants used in the treatment of diarrhoea. The study showed that there is immense potential for ethno-botanical research which would benefit not only the scientific world but also open up new avenues for augmenting the tribal economy through sustainable approach. The

study has revealed that the *Zeme (Naga)* tribes' knowledge about the medicinal use of plants is vast, unique and genuine. This knowledge is a guarded secret and is handed down by the traditional healer (*Hereukat eleupeu*) to any of the son of his choice, or to a daughter if absent. So based on the above observations ethnobolanical studies of Zingiberoideae from NE India was carried out.

2.3 Cytological studies

Chromosomal data have been underutilized in phylogenetic investigations despite the obvious potential that cytogenetic studies have to reveal both structural and functional homologies among the taxa. In large part this is associated with difficulties in scoring conventional and molecular cytogenetic information for phylogenetic analysis. Mitotic chromosomes offer a unique opportunity to observe the nuclear genome by microscopic means, allowing scrutiny of its components individually, as well as globally (the karyotype). Chromosome numbers of the few genera of Zingiberaceae like *Curcuma*, *Zingiber*, *Globba*, *Boesenbergia* have been studied. Chromosome atlas of the flowering and cultivated plant is available (Darlington & Wylie, 1955; Darlington & Ammal, 1945). In 1943, first paper on cytology of the Zingiberaceae in relation to their taxonomy was published (Raghavan & Venkatasubban 1943). They determined the chromosome numbers of 24 species but since their technique was based mainly on the sectioning method, very little data regarding the chromosome morphology was given. Ramachandran et al (1969) carried out the cytology of 27 species falling under 11 genera of the Zingiberaceae. The lowest chromosome number observed in South Indian plants of this family was 2n=22 and the highest 2n=96. New basic numbers observed include 25 for *Boesenbergia* and 21 for *Hitchenia*. The locally cultivated clone of the ginger plant (*Zingiber officinale*) showed evidence of structural hybridity resulting from inversions and interchanges. *Zingiber macrostachyum* also showed evidence of heterozygosity due to inversions.

The chromosome numbers of 22 species belonging to 10 genera of Zingiberaceae distributed in Thailand were investigated (Eksomtramage et al 2001). The somatic numbers range from 20 to 48 showing diploidy and polyploidy. Ten of these species were firstly reported, i.e. *Alpinia purpurata* (Vielli) K. Schum. (2n = 48), *Boesenbergia* aff. *rotunda* (2n = 20), *Cornukaempferia aurantiflora* J. Mood &

K. Larsen (2n = 46), *Curcuma* aff. *oligantha* Trimen (2n = 42), *C. rhabdota* Sirirugsa & M.F. Newman (2n = 24), *Etlingera elatior* (Jack.) R.M. Smith (white form) (2n = 48), *E. hemisphaerica* (Bl.) R.M. Smith (2n = 48), *Hedychium gomezianum* Wall. (2n = 34), *H. longicornutum* Bak. (2n = 34) and *Zingiber* aff. *wrayi* (2n = 22). Chromosome numbers of 11 species of Zingiberaceae from Thailand was determined (Saensouk and Saensouk, 2004). *K. angustifolia* was found to be diploid species with chromosome number of 2n= 22.

Skornickova et al (2007) investigated chromosomal and genome size variation in the majority of *Curcuma* species from the Indian subcontinent (Skornickova et al 2007). Six different chromosome counts (2n = 22, 42, 63, 70, 77 and 105) were found, the last two representing new generic records. Chromosome counts and genome size of three species *Hitchenia caulina, Kaempferia scaposa* and *Paracautleya bhatii* corresponded well with typical hexaploid (2n= 6x = 42). Forty three taxa representing a total of nine genera of Zingiberaceae found in South India were studied for their detailed karyomorphology (Joseph et al 2010). The chromosome spectrum in South Indian Zingiberaceae ranges from 2n=18 to 2n=63 with majority of the species concentrated in the number 2n=48 followed by 2n=63 and 2n=22.

No literature is available on the chromosome number studies of sub family Zingiberoideae especially native to NE India. In the present study an investigation on chromosome number in germplasms of the subfamily Zingiberoideae is attempted.

2.4 Molecular techniques assisted diversity and phylogenetic studies

There are two approaches for assessment of genetic variation in plants. One is the comparison of gene sequence at specific loci and the other involves molecular marker methods. The techniques that are used in plant identification are designed to detect the presence of specific DNA sequences or combination of sequences that uniquely identify the plant. Recently several papers have used molecular data to explore the phylogenetic relationships within the family Zingiberaceae (Wood et al 2000; Kress et al 2002) as well as within several genera (*Hedychium*: Wood et al 2000). These analyses have succeeded in clarifying the patterns of evolutionary relationships to

varying degrees. The phylogeny of *Hedychium* J. Koenig was estimated using sequence data of internal transcribed spacer regions 1 and 2 (ITS1, ITS2) and 5.8S nuclear ribosomal DNA. Sequences were determined for 29 taxa, one interspecific hybrid of *Hedychium* and one species in each of 16 other genera of *Zingiberaceae* representing tribes *Hedychieae, Globbeae, Zingibereae* and *Alpinieae*. Cladistic analysis of these data strongly supports the monophyly of *Hedychium*, but relationships to other genera are poorly supported (Kress et al 2002). Within *Hedychium*, four major clades are moderately supported. These clades are also distinguishable on the basis of number of flowers per bract and distribution. *Stahlianthus*, *Curcuma*, and *Hitchenia* also form a strongly supported clade. Based on this limited sample, *Zingiberoideae* was found to be polyphyletic. Based on the nuclear internal transcribed spacer (ITS) and plastid *matK* regions data of 104 species belonging to 41 genera of Zingiberaceae, Kress et al (2002) propose a new classification of the Zingiberaceae that recognizes four subfamilies and four tribes: Siphonochiloideae (Siphonochileae), Tamijioideae (Tamijieae), Alpinioideae (Alpinieae, Riedelieae), and Zingiberoideae (Zingibereae, Globbeae).

2.4.1 Molecular markers

A molecular marker is a DNA sequence that is readily detected and whose inheritance can be easily monitored. The use of molecular markers is based on naturally occurring DNA polymorphism, which forms the basis for designing the strategies to exploit for other purposes. A marker must be polymorphic; that is it must exist in different forms so that chromosomes carrying the mutant gene can be distinguished from the chromosome with the normal gene by a marker. Genetic polymorphism is defined as the simultaneous occurrence of a trait in the same population of two or more discontinuous variants or genotypes. The first such DNA markers to be utilized were fragments produced by restriction digestion-the restriction fragment length polymorphism (RFLP) based genetic markers. Consequently, several marker systems have been developed. One of the main aspects of this technology is that linkage between molecular markers and the traits of interest can be detected in a single cross (Chawla 2003). With morphological and biochemical markers, a separate cross is

required to test linkage with each new marker. A molecular marker should have some desirable attributes:

- It must be polymorphic as it is the polymorphism that is measured for genetic diversity studies
- Codominant inheritance: The different forms of a marker should be detectable in diploid organisms to allow discrimination of homo and heterozygotes.
- A marker should be evenly and frequently distributed throughout the genome.
- It should be easy, fast, and cheap to detect.
- It should be reproducible
- High exchange of data between laboratories.

Unfortunately, no single molecular marker meets all these requirements. A wide range of molecular techniques is available that detect polymorphism at the DNA level. These have been grouped into the following categories based on the basic strategy as non-PCR based approaches and PCR based approaches.

PCR-based techniques: Randomly Amplified Polymorphic DNA (RAPD), Microsatellites or Simple Sequence Repeat polymorphism (SSRP), Amplified Fragment Length Polymorphism (AFLP), Arbitrary Primed PCR (AP-PCR), etc. Collectively they are called as arbitrarily amplified DNA (AAD) markers. Under this thesis purview only PCR based molecular markers have been dealt. So the basics of RAPD, ISSR and AFLP techniques are discussed below.

During the last two decades thousands of studies have utilized AADs in plant science for various purposes (Bussell et al 2007). An informal search using Google Scholar (upto 20.12.2013) to obtain a rough estimate of how many studies have utilized AAD markers was performed and compared the values to those obtained for the other marker types reviewed here. The percentages presented in our pie chart (Fig 2.8) should be interpreted with caution, but it seems that AADs are still popular techniques.

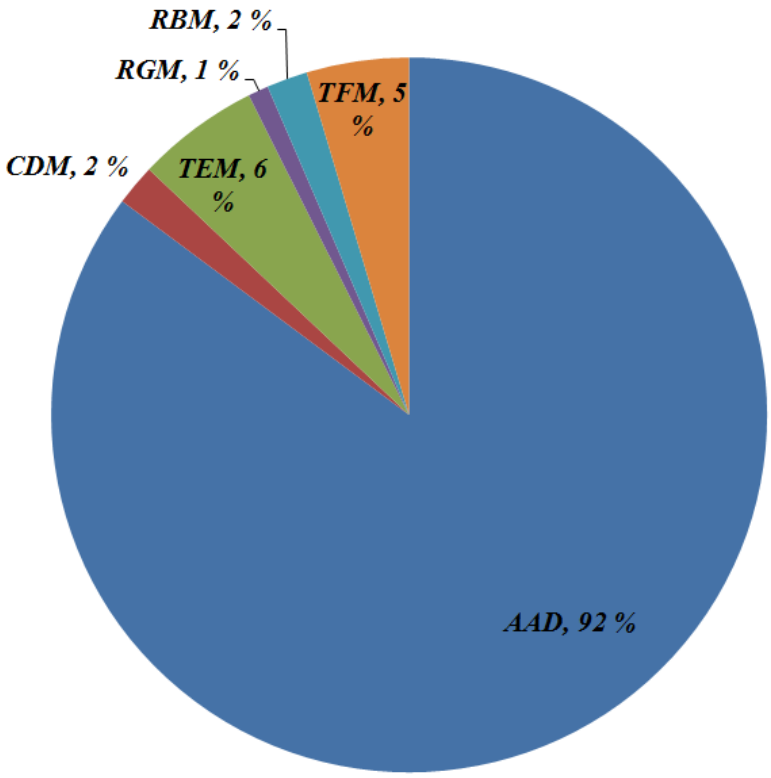

Fig 2.8 Pie chart representing usage of molecular markers. The diagram is prepared based on a google scholar search. (Abbreviations are as follows: AAD – Arbitrarily amplified DNA markers, including RAPD, ISSR, and AFLP. CDM – conserved DNA based markers.TEM – transposable element based markers. RGM – resistance-gene based markers; RBM – RNA-based markers, TFM – targeted fingerprinting markers.

2.4.2 Random Amplified Polymorphic DNA (RAPD)

RAPD is a PCR-based molecular marker technique where single short oligonucleotide primers are arbitrarily selected to amplify a set of DNA segments distributed randomly throughout the genome. This refers to DNA amplification using single random primers and shares the same principle with some differences in the experimental details. Of these, RAPD become available and is most commonly and frequently used. Willams et al (1990) showed that the differences as polymorphisms in the pattern of bands amplified from genetically distinct individuals behaved as Mendelian genetic markers. Welsh and McClelland (1990) showed that the pattern of amplified bands so obtained could be used for genomic fingerprinting.

2.4.3 Microsatellite directed PCR: anchored primers (ISSR)

This method is known variously as anchored microsatellite primed PCR (AMP-PCR), inter microsatellite PCR, and inter-SSR amplification (ISA or ISSR). In this approach, radiolabelled di- or trinucleotide repeats (SSR primers) are modified by the addition of either 3´or 5´ anchor sequences of 2-4 nucleotides composed of nonrepeat bases. The anchored primers can be $(CA)_8RG$, $(CA)_8RY$, $(CA)_8RTCY$ (where R=purine, Y= pyrimidine). One of the use of the ISSR primers was for the first strand cDNA synthesis of with oligo-d(T) primers carrying one or two non-T bases at the 3´ end. The anchor in the primer serves to fix the annealing of the primer to a single position at each target site on the template, such that every new polymerization event initiates at the same target position. Thus, there is little or no chance for primer slippage on the template. It has been reasoned that since dinucleotide repeats are most abundant in eukaryotic genomes than other types of microsatellites and at least (CA)n: (GT)n repeats appear to be ubiquitously represented in genomes, a small defined set of primers representing these repeats can be developed that would produce complex fingerprints.

2.4.4 Amplified Fragment Length Polymorphism (AFLP)

This is a highly sensitive method for detecting polymorphism throughout the genome and is becoming increasingly popular. It is essentially a combination of RFLP and

RAPD methods, and is applicable universally and is highly reproducible. It is based on PCR amplification of genomic restriction fragments generated by specific restriction enzymes and oligonucleotide adaptors of few nucleotide bases (Vos et al 1995). It is a novel DNA fingerprinting technique. DNA fingerprinting involves the display of a set of DNA fragments from a specific DNA sample. Fingerprints are produced without prior sequence knowledge, using limited set of genetic primers. AFLP technique uses stringent reaction conditions for primer annealing and combines the reliability of the RFLP technique with the power of PCR technique. In principle, AFLP involves the following steps:

1. DNA is cut with restriction enzymes (generally by two enzymes), and double-stranded (ds) oligonucleotide adapters are ligated to the ends of the DNA fragments.
2. Selective amplification of sets of restriction fragments is usually carried out with ^{32}P-lebelled primers designed according to the sequence of the adaptors plus 1-3 additional nucleotides. Only fragments containing the restriction site sequence plus the additional nucleotides will be amplified.
3. Gel analysis of the amplified fragments. The amplification products are separated on highly resolving sequencing gels and visualized by autoradiography. Fluorescent or silver staining techniques can be used to visualize the products in cases where radiolabelled nucleotides are not used in the PCR.

2.4.5 Comparison of the techniques for phylogenetic analysis at different level

The type of molecular method used to measure genetic diversity in plants will vary depending on the magnitude of genetic differences being assessed (Table 2.1). The analysis of data and interpretation in the measurement of genetic relationship between plants is more complex. The reliability of the conclusions of these studies is often dependent upon the rigour of the data analysis and interpretation. Phylogenetic studies and analysis of population genetics both require careful data interpretation. All methods require certain basic assumptions and have strengths and weaknesses. Mathematical methods have been developed to allow correction for the errors in estimation of genetic distance associated analysis and for reproducibility problem.

The relative information content of molecular markers can be compared by calculation of polymorphism information content (PIC, Anderson et al 1993)

$$PIC = 1 - \sum_{j}^{n} p_{ij}^2$$

where p_{ij}= the frequency of the *j*th pattern for marker *i* summed over n patterns.

2.4.6 PCR based molecular markers analysis in sub family Zingiberoideae

Use of PCR based techniques to study the genetic relationship of the subfamily Zingiberoideae is not very old (Vanijajiva et al 2005; Syamkumar & Sasikumar 2007; Gao et al 2008). Sigrist et al (2011) studied the genetic diversity among 39 turmeric germplasm originating from Brazil using SSR markers. Overall genetic variability for the groups studied, represented by Shannon-Weiner indexes, was found to be relatively low. Analyses of molecular variance (AMOVA) demonstrated major differences in turmeric varieties found in different countries (63.4%) and that most of the genetic diversity in Brazil is found within states (75.3%).

The molecular characterization of 15 economically important *Curcuma* species from the field gene bank of Indian Institute of Spice Research, Calicut was the first attempt by using ISSR/RAPD markers (Syamkumar & Sasikumar 2007). Thirty-nine RAPD primers yielded 376 bands of which 352 were polymorphic and out of the 91 bands produced by the 8 ISSR markers, 87 were polymorphic. Cluster analysis placed the 15 species into 7 groups that are somewhat congruent with classification based on morphological characters. The study also warrants the limitations of the conventional taxonomic tools for resolving the taxonomic confusion prevailing in the genus and suggests the need of molecular markers in conjunction with morpho-taxonomic and cytological studies while revising the genus, which is currently in progress. The maximum molecular similarity observed between two of the *Curcuma* species namely *C. raktakanta* and *C. montana* is suggestive of the need for relooking the separate status given to these two species. Further, the status of *C. montana* and *C. pseudomontana*, the two species mainly discriminated based on the presence of sessile tubers also need to be reassessed.

Table 2.1 Molecular methods suitable for different levels of genetic distinction†

	Population genetics to phylogenetic analysis					
	Individual	Variety	Species	Genus	Family	Higher levels
SSR	**	***	*			
RAPD	**	***	**	*		
RFLP						
Nuclear gene	**	***	**	*		
Mitochondrial gene		*	**	**		
Chloroplast gene		**	***	**		
Sequencing						
ITS		**	***	**	*	
Ribosomal genes (18 S)			*	**	***	***
Chloroplast gene				*	**	***
Less conserved proteins	*	**	***	**	*	
Conserved proteins			*	**	***	***

† Numbers of asterisks indicate increasing use of the method

The relationships among 19 accessions of Zingiberaceae belonging to 11 species of *Boesenbergia*, six species of *Kaempferia*, and two species of *Scaphochlamys* from Southern Thailand were studied using RAPD (Vanijajiva et al 2005). Two clusters were observed by dendrogram and PCA analysis. The first cluster consisted of the species from *Boesenbergia* and *Scaphochlamys* and the second cluster consisted of species only from *Kaempferia*.

Chinese *Hedychium* was grouped into two clusters based on SRAP markers (Gao et al 2008). The first group consisted of *H. densiflorum, H. convexum, H. spicatum* and *H. yunnamense*. The second cluster consisted of three sub-groups of 18 species. The highest similarity percentage (97 %) was reported between *H. flavescens* and *H. chrysoleucum*. The genetic relationships among nine species of *Curcuma* by 12 RAPD, 19 ISSR, and 4 AFLP primer combinations revealed the populations had been segregated based on topology. Dice genetic distanceThere is no such report available from the NE which gives a comprehensive data on intra-species and inter-

species variation in sub family Zingiberoideae. Although reports are available on many Zingiberaceae members, still little work has been done on the members of Zingiberaceae from NE India for adequate taxonomic profiling of this family.

2.5 Nuclear DNA content

The initial attempts to determine DNA amounts in cell nuclei (Caspersson & Schultz 1938) preceded the invention of its pivotal role in heredity. Shortly after, a constancy of DNA amount per organism was established, the term `C-value´ was coined by Swift (1950) which is DNA content of an unreplicated haploid chromosome complement (n). Hence, a nucleus in G1 phase of the cell cycle, with two copies of unreplicated genome has a 2C DNA amount (Fig 2.9).

Subsequently, it was found that there was no relationship between DNA C-value and organismic complexity (Mirsky & Ris 1951). The lack of correlation was later termed 'C-value paradox' by Thomas (1971). The discovery of non-coding DNA provided a clue to the paradox, but the origin, function and significance of variation in DNA content remain enigmatic. Several theories have been proposed to explain the C-value enigma' (Gregory 2001). Scientific disciplines, which profit from the knowledge of C-values, are numerous and include molecular biology, systematics and ecology (Bennett et al 2000). Despite its importance, C-values are known for only a fraction of all plant species, e.g. only 1.4 % of angiosperms (Hanson et al., 2003), and are easily accessible online (http://www.rbgkew.org.uk/cval/homepage.html).

2.5.1 Nuclear DNA content: concepts and symbols

The various terminologies followed for the nuclear DNA content measurements are as follows:

***Haploid number (n)*:** It is the number of chromosomes in a gamete. A somatic cell has twice that many chromosomes (2n).

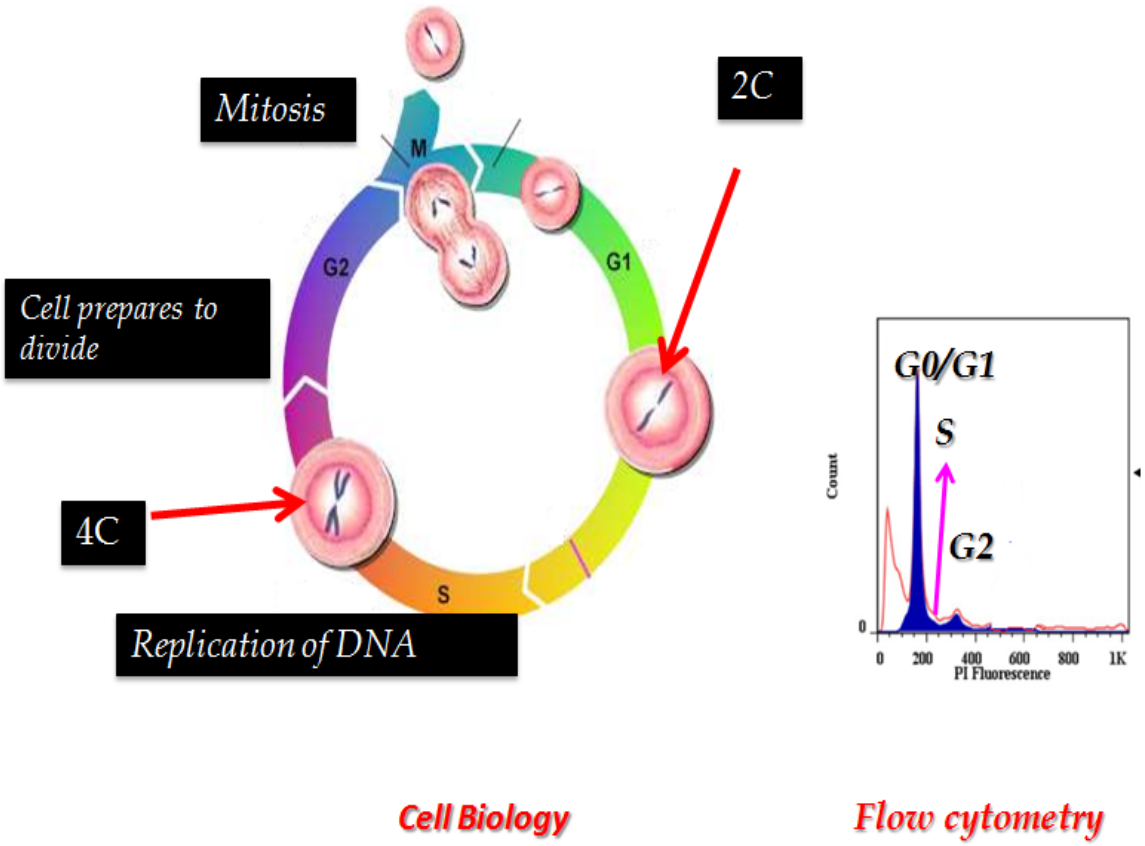

Fig 2.9 Nuclear DNA cotent: A Cell Biology-Flow cytometric interface

Monoploid number(x) : The number of chromosomes in a single (non-homologous) set is called the monoploid number (x), and is different from the haploid number (n). The abbreviated term for monoploid genome is C_X.

Holoploid genome size: is a shortcut for the DNA content of the whole chromosome complement characteristic for the individual (and by generalization for the population, species, etc.) irrespective of the degree of generative polyploidy, aneuploidies, etc. The abbreviated term for holoploid genome size is C-value (Obermayer & Greilhuber 1999 and Leitch et al 2005).

'Genome size' is often used for the DNA content of the monoploid genome or chromosome set. A summary of the key terminologies are presented in Table 2.2.

Table 2.2 Terminologies to express genome size

Genome status	Monoploid	Holoploid
Chromosome number designation	x	n
Covering term for genomic DNA content	Genome size	Genome size
Kinds of genome size	Monoploid genome size	Holoploid genome size
Short terms	Cx- value	C-value
Short terms quantified	1Cx, 2Cx, etc	1C, 2C, etc.

2.5.2 Methodology available for determination of nuclear DNA content

The first generation of researchers quantifying DNA amounts struggled with the lack of suitable approaches and instruments and largely used biochemical methods, which produced average values from many cells. Progress was made by the introduction of Feulgen microspectrophotometry, which provided a tool to measure DNA amounts in single cells (Greilhuber et al 2007). This method was widely adopted and led to the generation of genome size data for a vast number of species (Dolezel & Bartos 2005). The spectrum of methods suitable for plants expanded considerably in 1983, when a protocol for measuring DNA content by flow cytometry was developed (Galbraith et al 1983). The method was shown to be convenient and rapid and has enabled large-scale studies to be undertaken, the scope of which would have been inconceivable using other methods.

2.5.3 Flow cytometry

The ability to discriminate and quantify distinct populations of cells/ cell organelles has been increasingly important with the growing trend to focus biological studies in different cell types. Flow based cytometry and cell sorting are unique techniques that permit the identification, analysis and purification of cells based on their expression of specific markers. A flow cytometer and its different components have been shown (Fig 2.10). The light source is the blue, argon-ion laser that passes through a focusing lens. The forward scatter detector detects light scatter in a forward direction. A collecting lens is placed at right angle to the laser beam. A series of dichroic mirrors select out light of different wavelengths.

2.5.4 Units for presenting DNA amounts and their conversion factors

Nuclear DNA content can be presented relative to the DNA content of biological standard nuclei (% ratio), as mass units (usually picograms, pg) or as number of base pairs (bp, Mbp, Gbp). The amount of nuclear DNA of the unknown sample is calculated as follows:

$$Sample\ 2C\ value\ (DNA\ pg\ or\ Mbb) = \frac{Sample\ 2C\ mean\ peak\ position}{Reference\ 2C\ mean\ peak\ position} X\ Reference\ 2C\ value$$

A derivation of the factors has been published (Dolezel et al 2003), which is as follows:

$$DNA\ content\ (bp) = (0.978 \times 10^9)\ X\ DNA\ content\ (pg)$$

$$DNA\ content\ (pg) = DNA\ content\ (bp)/\ (0.978 \times 10^9)$$

2.5.5 Biological standards

Flow cytometer determines the relative fluorescence intensities. Data interpretations in terms of genome size necessitate a reference standard (i.e., sample with known nuclear DNA content). The standardization is either external or internal. The former involves successive analysis of the sample and the standard in the same voltage settings, whereas the latter involves co-processing (nuclei isolation, staining and acquiring) of nuclei both from sample and standard.

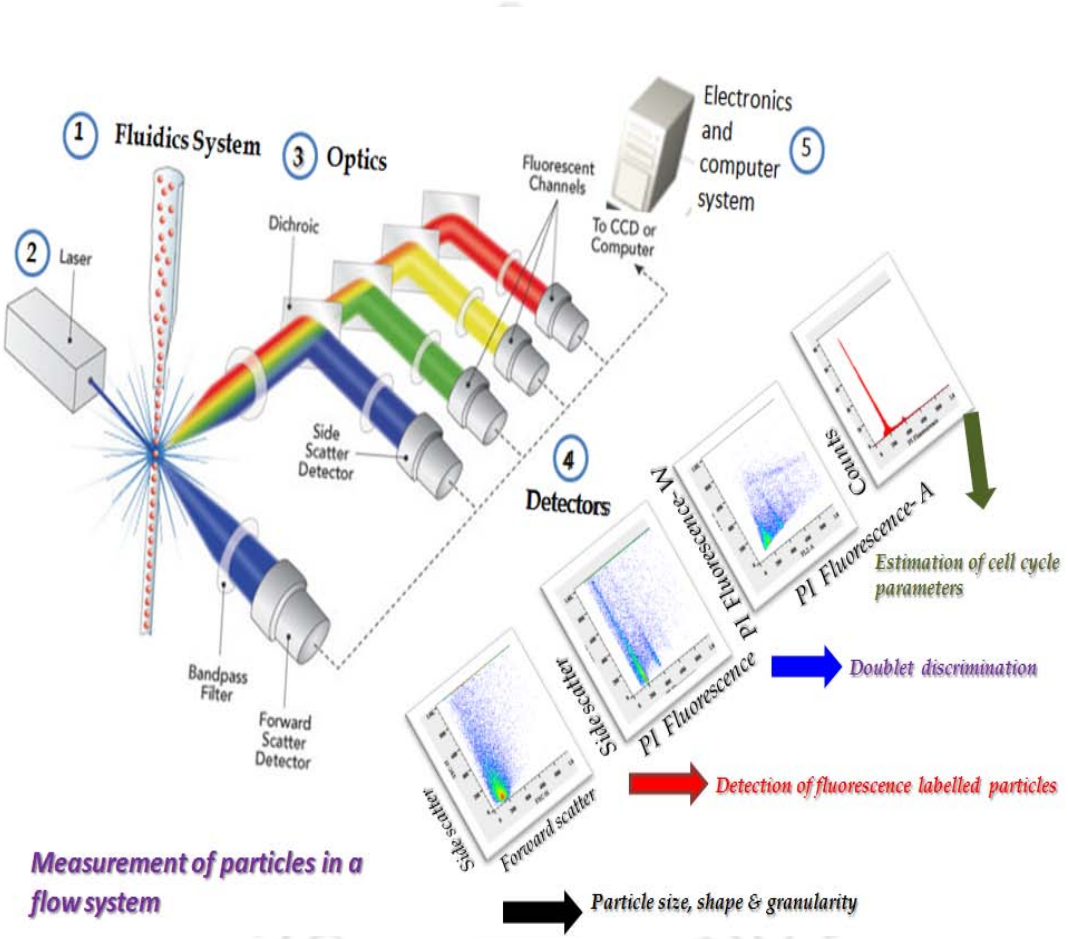

Fig 2.10 Flow Cytometer (BD-FACS CALIBUR) and its different components

The estimation of DNA content in absolute units needs internal standardization, and animal standards are not recommended (Greilhuber et al 2007). The proportion of literatures using internal standardization (91.8 %) was much higher than external standardization (6.1 %). In some papers it was found both methods are used (7.1 %). From all this reports it was interpreted that internal standardization was the most preferred method for researcher (Greilhuber et al 2007). Wide availability, cytological stability, absence of secondary metabolites, easy to grow in a wide range of geographical region is the primary requirement of an ideal calibration standard for nuclear DNA content study (Suda & Leitch 2010). Ability to produce high resolution, reproducible flow histogram and difference of less than 4 fold variation in nuclear DNA content of the unknown and the standard are the secondary requirements (Suda & Leitch 2010). The reference standards recommended for flow cytometric estimation of nuclear DNA content is shown in Table 2.3. The usage of the different reference standards for flow cytometric estimation of nuclear DNA content estimation has been given (Fig 2.11).

Table 2.3 DNA reference standards recommended for estimation of nuclear DNA amounts in absolute units

Sl no	Plant species and cultivars	2C DNA content (pg DNA)	1C Genome Size (Mbp)	References
1	*Oryza sativa* 'IR36'	1.01	493	Price and Johnston (1996)
2	*Solanum lycopersicum* L. 'Stupicke´ polnı´ rane´'	1.96	958	Dolezel et al. (1992)
3	*Zea mays* L. 'CE-777'	5.43	2655	Lysák and Doležel (1998)
4	*Pisum sativum* L. 'Ctirad	9.09	4445	Doležel et al. (1998)

Pisum sativum (Fabaceae; 14 %), *Hordeum vulgare* (Poaceae; 12%), *Petunia hybrid* (Solanaceae; 11 %) and *Zea mays* (Poaceae; 8 %) were the most popular standards used for the nuclear DNA content studies of angiosperms.

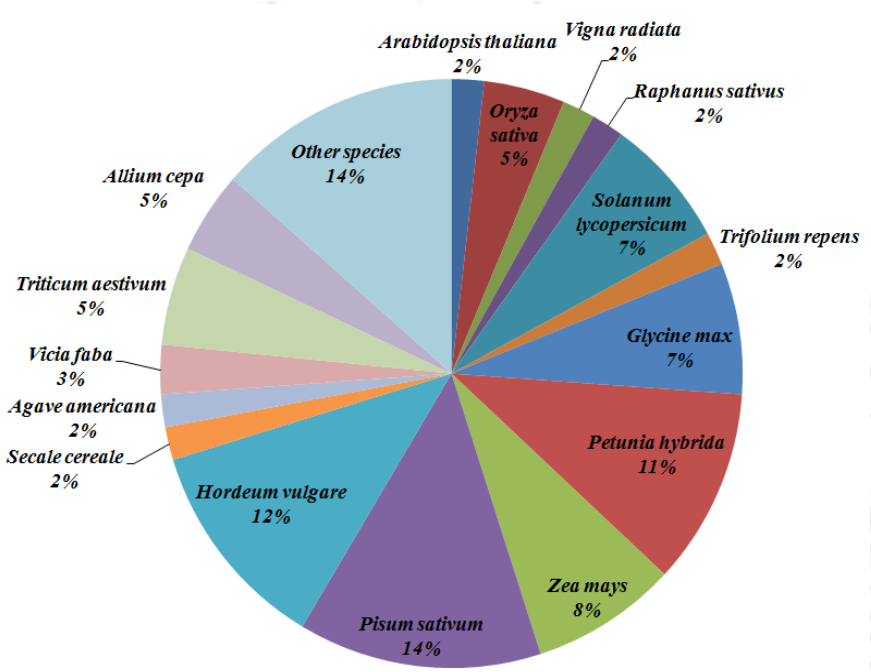

Fig 2.11 Pie diagram for the most popular plant reference standards used for flow cytometric estimation of nuclear DNA content from 1983 to 2013.

2.5.6 Sample preparation for flow cytometric measurement

2.5.6.1 Tissue selection

The choice of the optimum tissue type is a prerequisite of nuclear DNA content estimation. In most of the literatures fresh almost fully expanded leaves are used as material for flow cytometric studies (Greilhuber et al 2007). It is preferable to use colorless plant organs rather than those colored by anthrocyan (a fluorescence inhibitor) (Greilhuber et al 2007).The suitability of seeds as material for flow cytometric estimation has been investigated (Sliwinska et al 2005).

2.5.6.2 Isolation buffers

The method of preparing a suspension of nuclei for measurement follows the ingeniously simple procedure of Galbraith et al (1983). It consisted of chopping of plant material in isolation buffer with a sharp razor blades followed by sieving the homogenate to remove the tissue debris. The filtrate should be stained with a fluorochrome followed by flow cytometric estimation. The flow diagram of the flow cytometric estimation has been schematically shown (Fig 2.12).

The buffer should facilitate isolation of intact nuclei free of adhering cytoplasmic debris, maintain nuclei stability in liquid suspension and prevent their aggregation (Loureiro et al 2007). It ought to protect nuclear DNA from degradation and provide an appropriate environment for specific and stoichiometric staining of nuclear DNA, including the minimization of negative effects of some cytosolic compounds on DNA staining (Loureiro et al 2007). The commonly used buffer for flow cytometric estimation of nuclear DNA content has been shown (Table 2.4).

The literature survey of the usage of the buffer suggested two prevailing buffers (Galbraith and Commercial buffers) (Fig 2.13). It was found that there was no significant correlation between the selections of buffer with the plant developmental stage. Literature report suggests that Galbraith and LB 01 were used for the woody species, while Otto's, Arumuganathan Earle buffer and Tris-$MgCl_2$ were used for the herbs. There was only one report of the usage of hypotonic citrate buffer (Krishan 1975) in nuclear DNA content estimation for Angiosperms (Bharathan et al 1994).

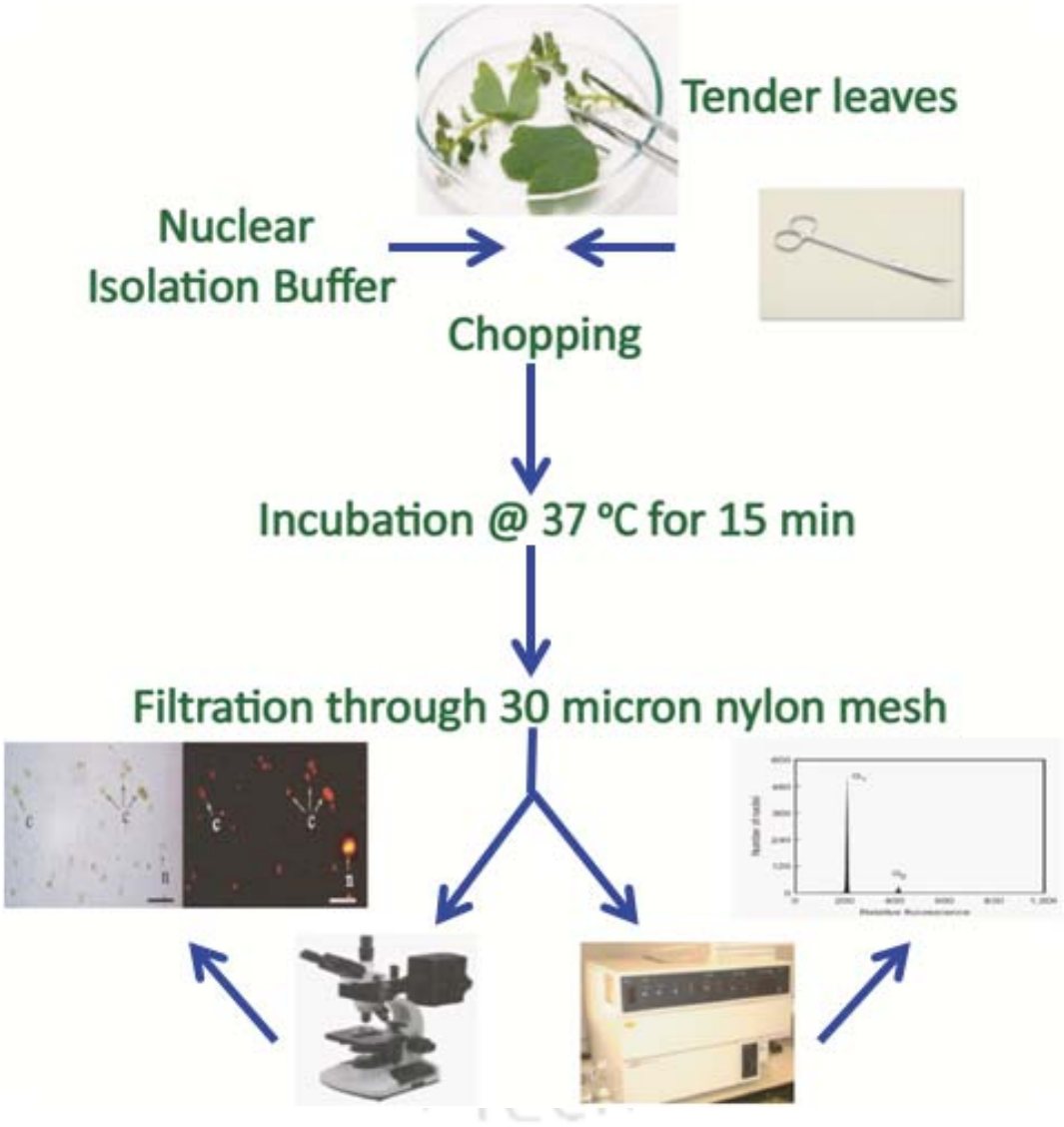

Fig 2.12 General flow cytometric procedure for estimation of nuclear DNA content.

Table 2.4 Composition of buffer for flow cytometric estimation of nuclear DNA content

Buffer	Composition	Reference
Galbraith's buffer	45mM MgCl2; 30mM sodium citrate; 20mM MOPS; 0.1%(w/v) Triton X-100; pH 7.0	Galbraith et al. (1983)
LB01	15 mM TRIS; 2 mM Na2EDTA; 0.5 mM spermine.4HCl; 80 mM KCl; 20 mM; NaCl; 15 mM b-mercaptoethanol; 0.1 % (v/v) Triton X-100; pH 7.5	Dolezˇel et al. (1989)
Arumuganathan and Earle	9.53 mM MgSO4.7H2O; 47.67 mM KCl; 4.77 mM HEPES; 6.48 mM DTT; 0.25 % (w/v) Triton X-100; pH 8.0	Arumuganathan and Earle (1991)
Marie's nuclear isolation buffer	50 mM glucose; 15 mM KCl; 15 mM NaCl; 5 mM Na_2EDTA; 50 mM ;sodium citrate; 0.5 % (v/v) Tween 20; 50 mM HEPES; 0.5 % (v/v) β-mercaptoethanol; pH 7.2	Marie and Brown (1993)
Otto buffers	Otto I buffer: 100mM citric acid; 0.5%(v/v) Tween 20 (pH approx. 2.3) Otto II buffer: 400 mM $Na_2HPO_4.12H_2O$ (pH approx. 8.9)	Otto (1990) Dolezˇel and Go¨hde (1995)
Tris-$MgCl_2$	200 mM TRIS; 4 mM MgCl2.6H2O; 0.5 % (v/v) Triton X-100; pH 7.5	Pfosser et al. (1995)
Baranyi's	Baranyi solution I: 100 mM citric acid mono hydrate 0.5 % Triton -X-100 Baranyi solution II: 400 mM Na2PO4.12H2O; 10mM sodium citrate; 25mM sodium sulfite	Baranyi & Greihuber (1995)
Bergounioux's	Tissue culture salts supplemented with 700 mM sorbitol; 1.0 % (v/v) Triton-X-100; pH 6.6	Bergounioux et al (1986)
Rayburn's	1mM hexylene glycol; 10mM $MgCl_2$; 0.5 % (v/v) Triton- X-100; pH 8.0	Rayburn et al 1989
Bino's	200 mM Manitol; 10 mM MOPS; 0.05 % (v/v) Triton-X-100; 10mM KCl; 10mM NaCl; 2.5 mM DTT; 10 mM Spermine.4HCl; 2.5mM $Na_2EDTA.2H_2O$; 0.05 % (w/v) sodium azide; pH 5.8	Bino et al (1993)
De Laat's	15 mM HEPES; 1 mM $EDTANa_2.2H_2O$; 0.2 % (v/v) Triton-X-100; 80mM KCl; 20mM NaCl;15 mM DTT; 0.5 mM Spermine.4HCl; 300 mM sucrose; pH 7.0	de Laat & Blaas (1984)

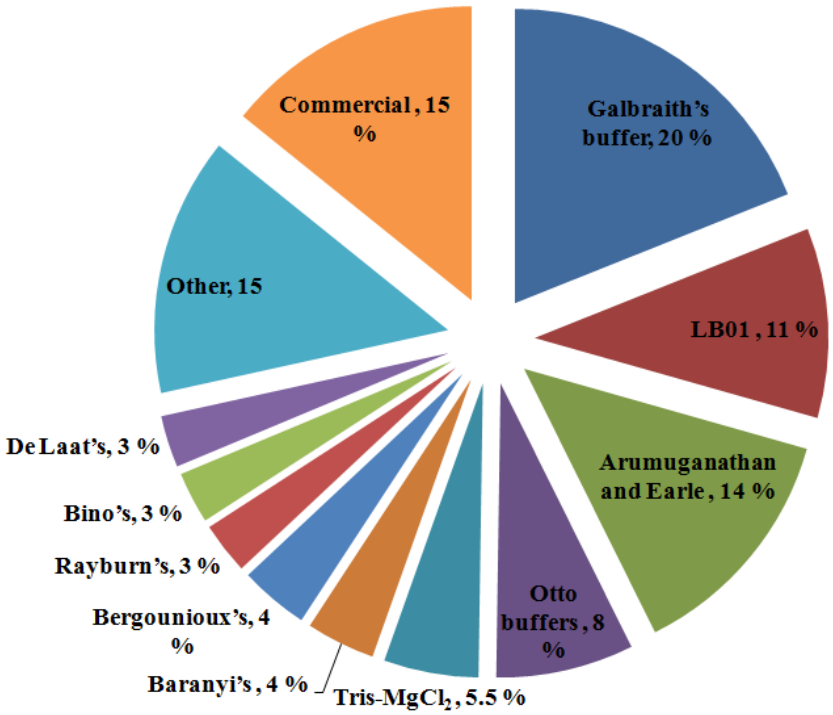

Fig 2.13 Pie chart representing the nuclear isolation buffer used for flow cytometric estimation of nuclear DNA content. Data obtained from 1982 to 2013.

2.5.6.3 Inhibitors

The most challenging problems faced by researchers has been adjusting the buffer composition to the requirements of specific species, especially if they contain staining inhibitors such as phenols, caffeine, and other secondary metabolites in the cytosol of their leaf cells (Price et al 2000). In spite of several studies, there is a lack of information on the mode of action of the staining inhibitors and no universal method to completely avoid their effects on DNA content estimation (Greilhuber et al 2007). Inhibition probably involves the intercalation of secondary metabolites into DNA and/or their direct reaction with the dye molecule that interferes with its fluorescence (Ramesh et al 2013). The addition of antioxidants, such as polyvinylpyrrolidone, β-mercaptoethanol, or dithiothreitol to a buffer, although helpful, does not always guarantee the correct measurement of DNA content (Fig 2.14).

2.7 Nuclear DNA content across Zingiberoideae

The availability of literature on nuclear DNA content estimation of Zingiberaceae are very few (Bharatan et al 1994; Das et al. 1999; Nayak *et al.*, 2006; Skornickova *et al.*, 2007). Nuclear DNA content of 62 species of angiosperms including 52 monocotyledons and ten dicotyledons has been estimated by flow cytometry using *Nicotiana tabacum* var. Xanthi as the internal standard (Bharathan et al 1994). The nuclear DNA content of *Curcuma zanthorrhiza* was estimated to be 2.60 pg. Das et al. (1999) used cytophotometry to study genome size in *C. amada* (4C = 3.120 pg), *C. caesia* (4C = 4.234 pg) and *C. longa* (4C = 5.100–5.263 pg). *C. longa* was also investigated by Nayak et al (2006), who observed 4C-values ranging from 4.30 to 8.84 pg in 17 varieties. The nuclear DNA content and chromosome number variation was studied for 46 *Curcuma* speices (Skornickova et al 2007) from Indian sub continent. Most of the species examined showed low intraspecific genome size variation (3.4% on average). There was no clear gap in nuclear DNA C-values between hexaploid and nonaploid plants and a major discontinuity in these cytotypes actually occurred between two groups of hexaploids. These results indicate that DNA content alone is often insufficient to distinguish between 6x and 9x cytotypes, and FCM measurements should always be accompanied by chromosome counts. The C-

values of diploid and high polyploidy taxa were more distinct, but again FCM measurements alone may not be an accurate indicator of ploidy level, as illustrated by 11-ploid *C. oligantha* and 15-ploid *C. raktakanta* with reverse total amounts of nuclear DNA. Nuclear DNA content was estimated for 42 *C. zedoaria* accessions from five ecologically distinct habitats of Bangladesh (Islam et al 2007). The nuclear DNA content was statistically different between individual populations ranging from 3.12 to 3.44 pg. They reasoned the variation in the nuclear DNA content is caused not due to aneuploidy but because of the heterochromatin variability.

Chandrmai et al (2012) studied nuclear DNA content vaiation of the *Kaempferia* species from Thailand. Their study revealed that the nuclear DNA content of *Kaempferia* varies from 2.33-9.73 pg. The results reveal that genome size of *Kaempferia* species study corresponded with chromosome number and leaf character. Nuclear DNA content of *Hedychium spicatum* was estimated by flow cytometry to be 2.94 pg taking *Zea mays L.* 'CE-777' as internal standards.

The tissue type variation of nuclear DNA content of Zingiberoideae species was not studied. The buffer Galbraith et al (1983) and the Otto (1990) buffer were explored for the flow cytometric estimation of nuclear DNA content of Zingiberoideae species. Nuclear DNA content variation across the family has not been studied so far.

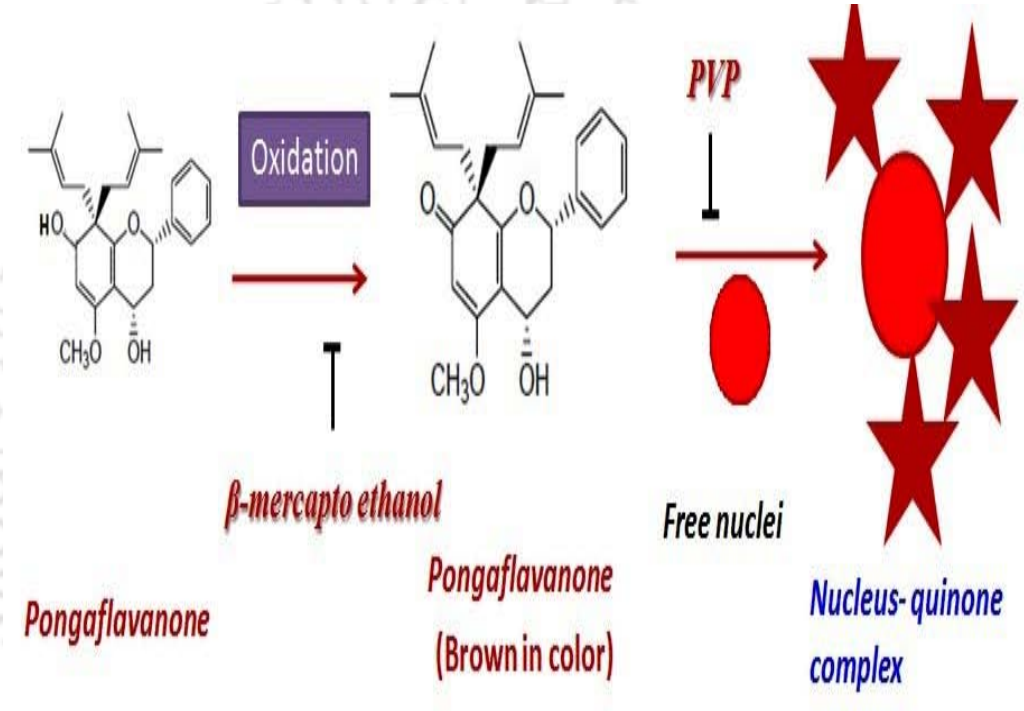

Fig 2.14 Mode of action of the stripping of inhibitors from *P. pinnata* by β-mercapto ethanoland polyvinyl pyrrolidones (Ramesh et al 2013).

CHAPTER 3

ETHNOBOTANICAL SURVEY AND KARYOMORPHOLOGICAL STUDIES OF ZINGIBEROIDEAE

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This chapter gives the work carried out in germplasm collection, maintenance and the ethno botanical information collected. The chapter also determines the chromosome number determination of the some of the members of the subfamily. Results of karyomorphological analysis has also been discussed.

ETHNOBOTANICAL SURVEY AND KARYOMORPHOLOGICAL STUDIES OF ZINGIBEROIDEAE

3.1 Introduction

The members of Zingiberoideae are important for their economic, medicinal and ornamental significance. In spite of the economic benefit of the family, several order of polyploidization (2x-15x) and hybridization created phylogenetic and taxonomic confusion (Skornickova et al 2007; Zaveska et al 2012) opening options for further work.

Ethno-botany is the study of the different uses of plants in day to day life of the ethnic communities (Srivastava et al 2010). Digging into local literature have pointed out that there are numerous medicinal plants described for treatment of many diseases and herbal medicine. However, there are few reports on ethno botanical study of Zingiberoideae of NE to be effective in a very wide range of diseases. So to overcome the knowledge gap between the science and folklore, an attempt has been made keeping in view the usefulness of members of Zingiberoideae sub family.

Chromosomal data have been underutilized in phylogenetic investigations despite the obvious potential that cytogenetic studies have to reveal both structural and functional homologies among taxa. In large part this is associated with difficulties in scoring conventional and molecular cytogenetic information for phylogenetic analysis. Mitotic chromosomes offer a unique opportunity to observe the nuclear genome by microscopic means, allowing scrutiny of its components individually, as well as globally (the karyotype). Chromosome numbers of the few genera of Zingiberaceae like *Curcuma*, *Zingiber*, *Globba*,and *Boesenbergia* have been studied (Skornickova et al 2007).In the present study an investigation on chromosome number and associated karyomorphometrical parameters in some selected species of the sub family Zingiberoideae has been attempted.

3.2 Methods

3.2.1 Collection of germplasm

Zingiberaceous both domestic and wild relatives were collected from their natural habitats across NE India and maintained in the Departmental Garden, Gauhati University (Table 3.1). The collections were made during the flowering season (April-August). Some of the representative photographs of each genushave been shown below (Fig 3.1)

3.2.2 Identification and maintenance of germplasm

The collected plants were maintained as both live specimen and as herbariums in the departmental green house of Indian Institute of Technology Guwahati (IITG), Assam. Herbaria are essential for the study of plant taxonomy, geographic distributions, and the stabilizing of nomenclature. The specimens in a herbarium are often used as reference material in describing plant taxa; some specimens may be types. To preserve their form and colour, collected plants were spread flat on sheets of newsprint and dried in between absorbent paper. The specimens were then mounted on sheets of stiff white paper, labelled with all essential data such as date and place found, description of the plant, altitude, and special habitat conditions. The sheet was then placed in a protective case. As a precaution against insect attack, the pressed plant was frozen or disinfected using spirit or 80% ethanol.

A botanical specimen consists of the whole plant, complete with roots, stem, leaves, flowers and if possible, with fruits. Since ZIngiberaceous species are large herbs, a portion of a twig with leaves and flowers were collected as a representative of herbarium. The size of the specimen used for preservation was set according to the size of the herbarium sheet (17x11 inches) used for mounting. After collection, the specimen was tied with a tag number. Most of the plants wilted very rapidly after being cut or dug out of the ground. To avoid this, the dugout plants and cut twigs were kept in polythene bags along with some soil retained with the rhizome. This enables the plants to study fresh for more than 6 hrs. The flowers of *Hedychium* were too large to be pressed with the leaves and were kept separately in small polythene bags. Two or three specimens were collected for every species. Soon after collection, pressing of the specimens was carried out with a ventilated drying press. While pressing, care was taken so that the leaves are upside down to show the ventral side.

Table 3.1 List of Zingiberoideae plants

S. No	Species name	Voucher no	Collected from	S. No	Species name	Voucher no	Collected from
1	*C. amada*	17746	Kahikuchi (Assam)	12	*H. coronarium*J. Koenig	17755	Guwahati (Assam)
2	*C. angustifolia*Roxb.	17745	Shillong (Meghalaya)	13	*H. dekianum* A.S.Rao & Verma	12868	Barpeta (Assam)
3	*C. aromatica*	17749	Kamrup (Assam)	14	*H.flavum* Roxb.	12909	Barpeta (Assam)
4	*C. caesia*Roxb.	17746	Goalpara (Assam)	15	*H. stenopetalam* Lodd.	12869	Ultapani (Assam)
5	*C.* domestica	12888	Nagaon (Assam)	16	*H. spicatum*Lodd.	17750	Panbazar (Assam)
6	*C. longa* L.	17765	Kamrup (Assam)	17	*H. chrysoleucum*Hook.	12851	Margherita (Assam)
7	*C. zedoaria*(Christm.) Roscoe	12859	Darrang (Assam)	18	*H. gardnerianum* Wall. ex Spreng.	17753	Bomdila (Arunachal Pradesh)
8	*K. galanga*L.	12871	Khetri (Assam)	19	*H. marginatum* Clarke	17754	Imphal (Manipur)
9	*K. angustifolia* Roxb.	17767	Jalukbari (Assam)	20	*H. flavescens* Lodd.	17752	Ziro (Arunachal Pradesh)
10	*K. pulchra* Ridl.	17766	Jalukbari (Assam)	21	*H. aurantiacum*Wall.	17751	Lamkangkhunou (Manipur)
11	*K. rotunda*Blanco.	12870	NEDFi (Assam)	22	*H.coccineum*Wall.	17756	Chandel (Manipur)

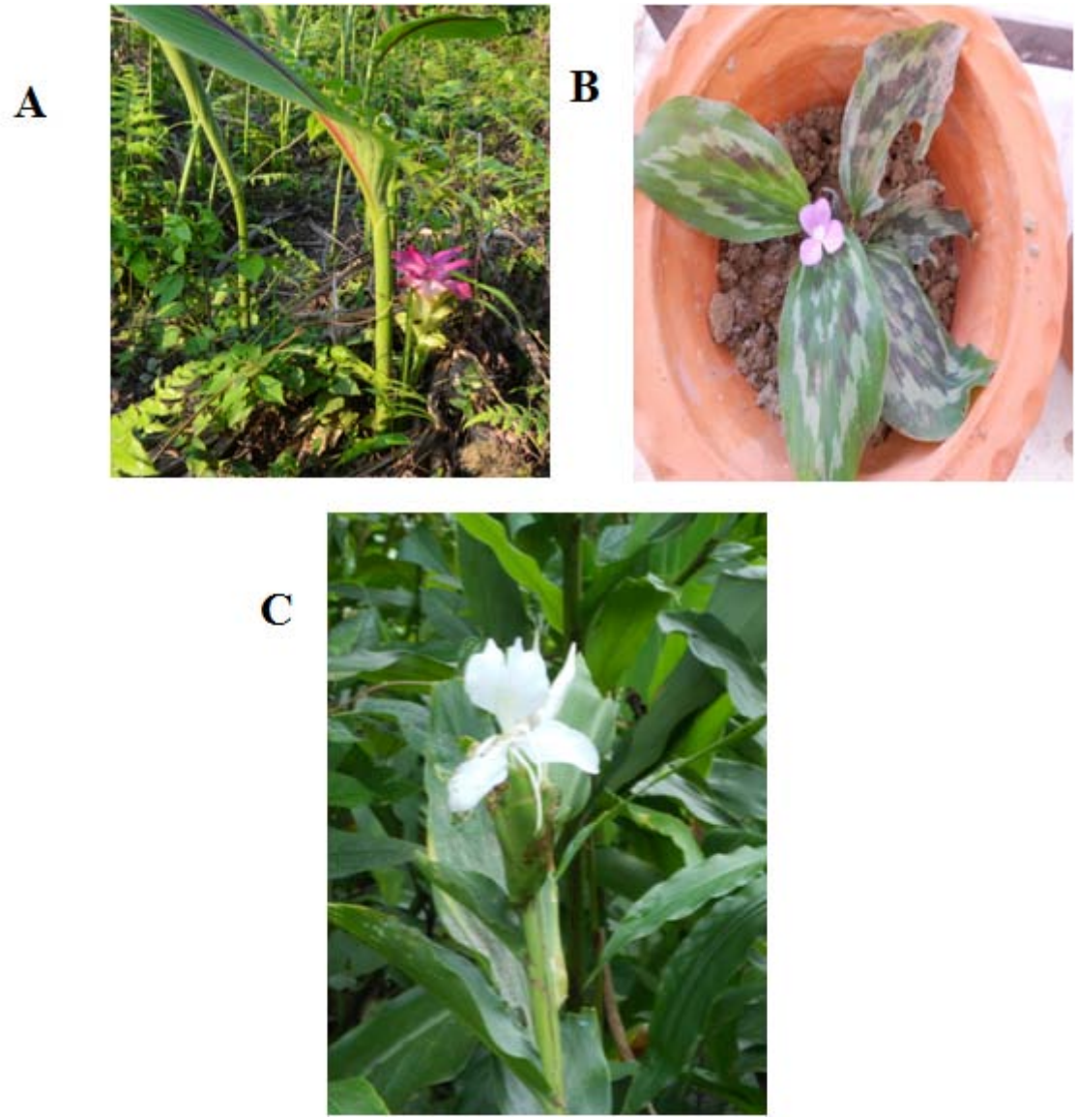

Fig 3.1 Representative taxa investigated. **A.** *C. zedoaria* (Christm.) Roscoe. **B.** *K. pulchra* Ridl. **C.** *H. coronarium* J. Koenig.

Rhizomes were difficult to press satisfactorily and hence were dried separately with tags. The specimens were examined in every 24 hrs. The newspapers were replaced with new ones every week. Next, the plant twig was mounted to a herbarium sheet and was labelled. A label consisted of collection number, Latin name, family name, vernacular name, habit, habitat, locality, date of collection and collector's name. The pressed herbarium sheets were subsequently stored under a cover (species cover). Collected specimens were identified by studying morphological traits following taxonomic rules and with the help of herbaria of Gauhati University (Fig 3.2).

The collected plants along with the rhizome and flowers were properly tagged and maintained in the departmental green house, IITG and botanical garden of Gauhati University (GU) (Fig 3.2). The features were also preserved as herbaria for future reference. The specimens were later identified by taxonomists at Botany Department GU, Assam. The botanical name was written as in International Plant Name Index (IPNI) database.

3.2.3 Ethno botanical survey

The current ethnopharmacological survey was conducted by making field trips to parts of NE during the period March 2008 to August 2009. Trips were made to villages and tribal areas during different season, so as to include wide number of species of the family. As the members of the family Zingiberaceae are known to bloom during monsoon season, a special account was taken to organize the visits during this season. Most of the Zingiberaceous plants are rare and some are endangered too. Moreover, many plants grew on slopes of hills so at times it was not only difficult but inaccessible too for collection and subsequent documentation. Since the choice of individual informant to be interviewed was of great importance to the reliability of the information, all contacts were established with elderly and local practitioners. We selected healers who utilized medicinal plants as therapeutic substance and treated patients outside their immediate circle of family and friends. Particularly in some cases they were the only known healer in the region. The healers were asked to identify the plant species from his collection as well as from natural habitat.

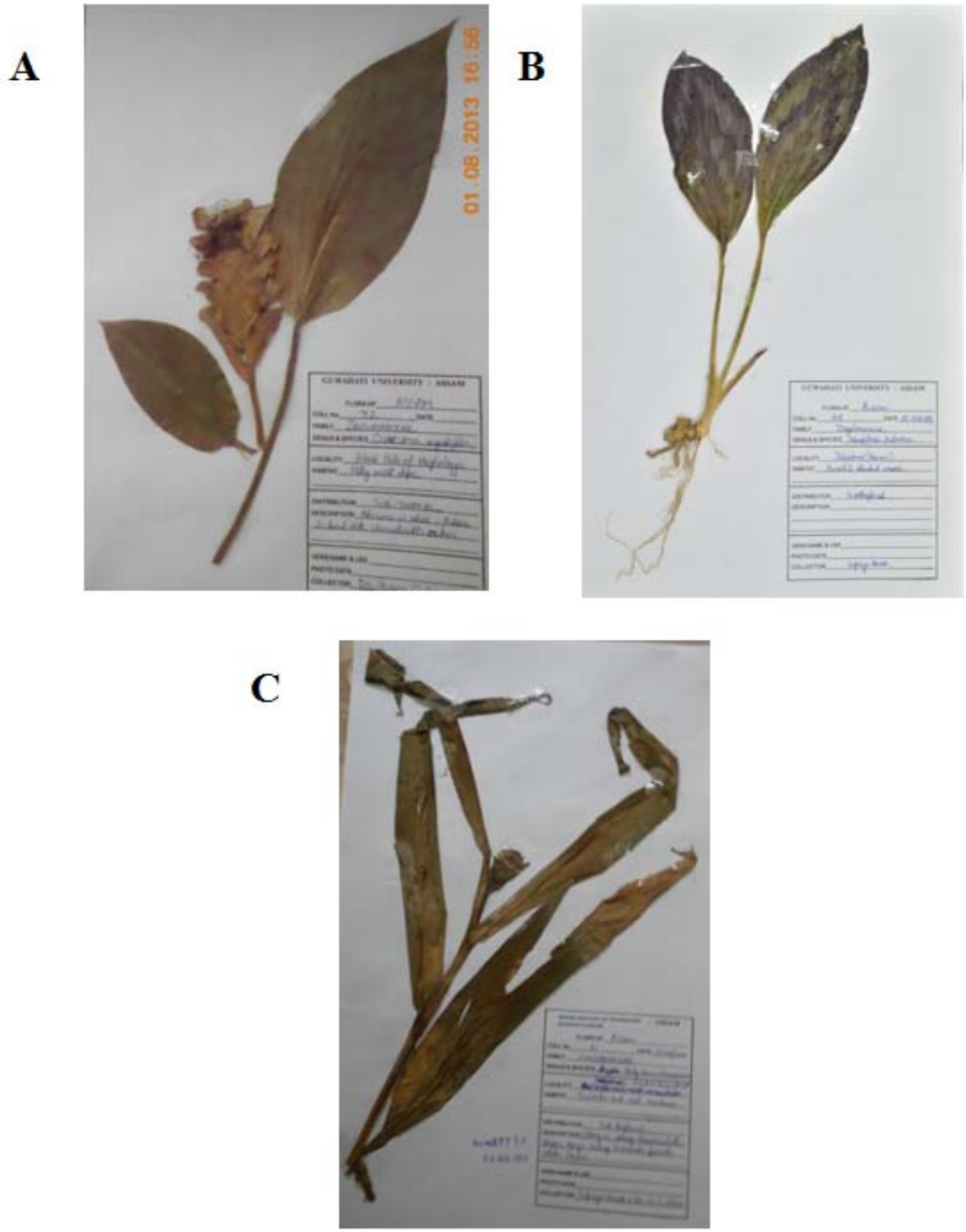

Fig 3.2 Maintenance of the germplasm in the form of herbarium. **A.** *C. angustifolia*Roxb.B., **B.** *K. pulchra* Ridl.**C.** *H. coronarium* J. Koenig

A structured interview forms was used to collect information in the local language and respondents were queried for the herbal remedy known to him for various diseases. The mode of preparation and administration were also recorded. The acquired data were also cross-checked for its pharmacological significance with the available literature. All doubtful and misleading cases where the informants showed little knowledge concerning the identification of plant species either in the field or from specimens were excluded from this survey and the information provided was discarded. A medicinal use was accepted as valid only if it was confirmed by at least two separate healers. The information obtained was documented in a tabular form. The data included the botanical name, local name, location, plant parts used, mode of preparation, administration and their utility as remedy for treating human diseases. The list of major ailments was compiled and the number of plants utilized in treatment was determined.

3.2.4Cytological analysis

3.2.4.1 Collection and storage of root tips

Conventional rapid squash method was followed to prepare the metaphase stage of cell division(Skornickova et al 2007)for each species of studied under investigation. Root tip collection from plant maintained in hydroponic culture was preferred in the morning hours (8AM to 11 PM). The washing of the root tips with distilled water followed by soaking in filter paper was done to remove the traces of water.

3.2.4.2 Pre-treatment and fixation

Pre-treatment of the root tips were carried out in saturated para dichloro benzene (PDB) solution at room temperature for 3 hours. After 3 hours of pre-treatment, the root tips were washed in distilled water several times to remove most of the PDB attached with the root tips followed by drying with the help of filter paper.

3.2.4.3 Fixation

Fixation is necessary to kill the material rapidly in such a way that the internal structures are preserved in a life-like form. In this study, the root tips were kept in fixative (Carnoy's fluid 3:1 absolute ethanol and glacial acetic acid) for 4 hours at 4^{o}C. The root tips were washed with 70% ethyl alcohol for 3-4 times and were preserved in 70% ethyl alcohol for further use.

3.2.4.4 Hydrolysis and staining

The root tips were hydrolysed with 1:1 mixture of 1N HCl and absolute ethanol at room temperature for 30 seconds.

3.2.4.5 Slide preparation, squash and observation

The root tips were immediately transferred to a slide containing one drop of lacto propionic orcein (1%). The root tip portion (deeply stained) was carefully cut with the blade and other portion was discarded. Cover slip was placed on the studied material and excess acid was soaked with filter paper. The cover slip was gently pressed with the thumb. The material was further heated again over the lamp to increase the intensity of the staining. Finally, the slide was observed under compound microscope at 10X and40X resolutions. All images were taken in the Olympus CX31 microscope fitted with Micropublisher 3.3 camera.

3.2.5 Estimation of karyomorphological parameters

Well spread metaphase plates were photographed using Micropublisher 3.3 camera fitted to camera attached to computer. The contrast of each image was increased to a satisfactory level by using Adobe Photoshop 5.5. The measurement of each chromosome length (in micrometer, µm) from enhanced image were done by loading the enhanced image to the IMAGE PRO PLUS software loaded in the computer attached to microscope. The chromosome were classified as A (0.99 to 0.7 µm), B (more than 0.5 µm and less than 0.95 µm) and C (more than 0.24 µm less than 0.49 µm) (Ravindran et al 2007). Apart from this, following numerical values were calculated for each investigated taxa.

1) Total chromosome length (TCL)
2) Average chromosome length (ACL)
3) The disparity index (DI) of chromosomes in a karyotype calculated by the formula as described by Mohanty et al 1991

$$DI = \frac{(longest\ chromosome - shortest\ chromosome)}{(longest\ chromosome + shortest\ chromosome} \times 100$$

4) The variation coefficient (VC) among chromosome complements determined by the formula given by Verma 1980.

$$VC = \frac{Standard\ deviation}{Mean\ length\ of\ chromosome} \times 100$$

3.3Result and discussion

3.3.1Collection of germplasm

Zingiberaceae germplasm were collected from the wild and also in the cultivated state from different locations of the NE India by undertaking field trips. The four field trips were carried out during the span of the thesis purview covering almost the entire sister states of NE India. The trips were particularly carried out in the rainy season which is the flowering time of Zingiberaceous plants. The plants were collected based on the inflorescence morphology and preliminary identification was being carried out on the genus and species status of the plants by field taxomists of each state university and/or institutes (Gauhati University for Assam trips, Institute of Bio resource and Sustainable Development for Manipur trips, Krishi Vigyan Kendra of Aizawl forMizoram trips, Jawaharlal Nehru College for Arunachal Pradesh trips).

3.3.2 Systematic identification of germplasm by morphological characters

Key to the Kingdom: Presence of chlorophyll, autotrophic, whole body is divided into stem, root and leaves. ------- **Plantae**

Key to the Division: Plants with flowers and seeds, ovules enclosed within the ovary --------- **Angiosperms**

Key to the Sub-division: Leaves simple with parallel venation, Flowers mostly trimerous, one cotyledon. ----- **Monocotyledons**

Key to the Order: Inflorescence racemose, flowers bisexual, zygomorphic, creeping horizontal or tuberous rhizomes. -------- **Zingiberales**

Key to the Family: Flowers zygomorphic, epigynous, only one fertile stamen, other stamens transform into staminodes. ------- **Zingiberaceae**

Key to the Sub-family: All parts aromatic, leaves distichously arranged in two rows, sometimes tufted or single, sheaths open on side opposite lamina, lateral staminoids usually petaloid or represented by a teeth at the base of the labellum, aromatic oil present ----------**Zingiberoideae**

Key to the Genus: Inflorescence central appear with leaves with a terminal plume of sterile bracts, peduncle enclosed within leaf sheaths, several flowers forming a spike,

each flower or each cluster of flowers is subtended by 2 bracts, reddish or pinkish white in colour, nectaries present. ----- ***Curcuma***

Key to the Genus: The roots are fibrous, fleshy with terminal globular to fusiform storage tubers. Leaves are either erect semi erect type or adpressed type. Ovaries are trilocular with axile placentation. ----- ***Kaempferia***

Key to the Genus: Terrestrial, epiphytic or epilithic, perennial herb. Pseudostems 0.4-2.4m high, leaf sheaths greenish or reddish, bladeless sheaths. Ligule oblong, 0.2-5.9 cm long, pubescent or glabrous, apex truncate-emarginate, acute, bilobed or rounded. Leaves lanceolate-oblong or elliptic, base cuneate, apex acute acuminate or caudate, margin entire to slightly undulate, upper surface glabrous, lower surface glabrous or pubescent. Inflorescence a terminal spike, erect,7.4-52.3 cm, glabrous or pubescent; peduncle 1.7-21.1 cm long; bracts imbricate elongated to visible rachis or not imbricate, rachis usually visible, apex acute or rounded, glabrous or pubescent, bracteole, folded or tubular, membranous, glabrous or pubescent, shorter or longer than bract. Flowers white, yellowish to golden yellow or reddish, fragrant; calyx tubular, split on one side, apex acute to 3-dented, glabrous or pubescent; corolla tube slender or twisted, glabrous or pubescent, 3-lobed, lobeslinear, apex hooded; lateral staminodes linear-oblong, oblanceolate, or elliptic; labellum ovate-elliptic, obovate or suborbicular-orbicular, base attenuate into claw, apex acute or emarginate to deeply divided; filament 0.7-7.4 cm long, shorter or longer than labellum; anther dorsifixed, base divaricate 0.3-1.8 cm long; ovary glabrous or pubescent, 3-loculed, placentation axile, epigynous glands 2; stigma densely ciliate, green. Fruits glabrous or pubescent, ellipsoid-oblong or globose. Seeds numerous, ellipsoid-oblong or globose, arilorange-reddish ----------***Hedychium***

3.3.3 Floral diagram

Floral diagrams are stylized cross sections of flowers that represent the floral whorls as viewed from above. Rather like floral formulas, floral diagrams are used to show symmetry, numbers of parts, the relationships of the parts to one another, and degree of connation and/or adnation. The floral diagram and floral formula of the three representative genera has been shown (Fig 3.3).

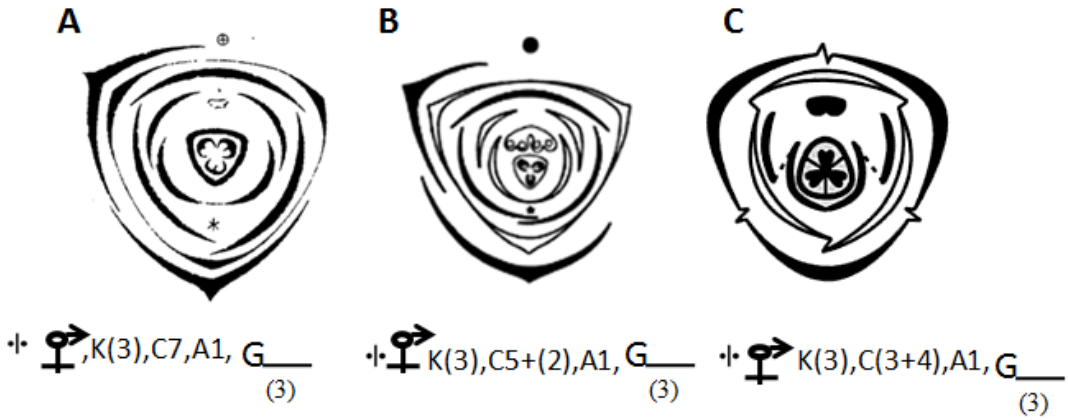

Fig 3.3 Floral diagram and floral formula of studied plants. **A.** *Curcuma* **B.** *Kaempferia* **C.** *Hedychium*

3.3.4 Ethno botanical survey of the subfamily Zingiberoideae in Northeast India

The survey involved 14 taxa belonging to 3 genera of the family Zingiberaceae from NE region of India. Most of the plant species grow naturally in the different regions and their properties are important in traditional herbal medicine. Among the states of NE region, Arunachal Pradesh (AP) was found to be floral rich as far as members of the family Zingiberaceae is concerned (88%). Plants like *C. longa* were found to occur in all the 7 sister states and has a wide geographical distribution. All the documented plants were found to be prevalent in use by local practioners for their therapeutic use against human disease and as herbal care as enlisted in Table 3.2. In general the medicinal plants were collected by the males and prepared by the females. Various parts of the plants are being used as herbal medicine although rhizome was the most frequently used plant part. This is well supported by the scientific literature as various molecules and extract from the rhizome have showed bioactivity (Ficker et al 2003; Lantz et al 2005; Chien et al 2008; Kumar & Singh 2008; Lee et al 2009). Some medicinal preparations were essential oils extracted from various plants parts such as *C. angustifolia*, *C. caesia* showing antifungal activity (Banerjee & Nigam 1976; 1977). The essential oils were a good source of fragrance apart from having the desired therapeutic use. Another thing that emerged out from the survey was that the plants were used by the practitioner singly or in association in different proportions for treating human ailments. The remedies which involved merely the use of single plant could be of great interest for the development of novel drugs as the exploration of therapeutic activity bearing ingredients from a single plant may be easier (Saikia et al 2006).

The majority of herbal medicines were prepared in aqueous medium and the healers administer the remedies in various forms like decoction prepared by boiling plant parts in hot water, water extract, fried material, raw plant part, rhizome paste, milk pudding, oils etc. The remedies were administered orally or locally confined to the affected portion of the body according to the treated disease and preparation method. In addition to pure herbal preparations, in some cases the drug was administered along with milk, ghee, honey, curd etc. to enhance the effect of the herbal preparations or to make the preparations palatable.

In current survey, the use of only underground plant parts for medicinal purposes was found to be higher (56%) than the case where both above and underground parts were used together (29%). The usage of aboveground plant parts were less comparatively (only 15%). Rhizomes were used in most of the cases followed by fruits, leaves, shoot and flower. In one case viz. *H. coronarium* all plant parts were found to have a medicinal value.

Fourteen Zingiberoideae plants were reported to be used in curing about 25 types of ailments, of which the highest numbers of plants species were used for the treatment of gastrointestinal disorders followed by chest and lungs related diseases (Fig 3.4). The plants also showed effective activity as antipyretic, analgesic and anti-inflammatory. We observed that some plants were used for ailment like cardiac disorder, kidney and urinary disorder, skin related diseases, irregular menstrual cycle, diabetes and as abortifacient. A close analysis revealed that about 5 plants are known for their use to cure multiple disorders. The rhizome powder from some plants such as *C. aromatica* and *H. spicatum* are used as anti-venom for snake and insect bite. Mostly the rhizome as a whole or as paste is used for medication. In certain cases, preparation of medicine involved use of a mixture of herbs and sometime molasses. Thus it can be said that the discovery of different plant species belonging to subfamily Zingiberoideae used by different tribes and communities of NE India paves way to undertake a detailed ethnobotanical study of the region. The use of single plant as a therapy for an ailment was observed to be 84% which is a good indication for future research leading to drug discovery. The work has brought to light some hidden but popular prescriptions of ethnic group of the NE region. These new prescription will help mankind in short term, by providing improved phytotherapeutic preparations while an extensive pharmacological study will elucidate new drug molecule. Though some scientific literature is available for most of the species, the specific properties of all the species has not been studied yet. A further pharmacological study of these particular and scientifically unexplored properties of Zingiberaceae appears promising.

Table 3.2: List of plants and their ethanoobotanical information

Botanical name	Local name	Location	Parts used	Mode of preparation	Usefulness	Experimentally proven fact
C. amada	Amada (Assamese); Aiengpui (Mizo)	Arunachal Pradesh, Assam, Manipur, Mizoram	Rhizome	Rhizome extract and paste.	Carminative, bronchiolytic and vulnerary	Anti-inflammatory (Mujumdar et al., 2000), Cholesterol lowering activity (Srinivasan et al 2008) Antioxidant and Antibacterial (Policegoudra et al 2007)
C. angustifolia	Yaipan (Manipuri); Gorusat haladhi (Assamese)	Arunachal Pradesh, Assam, Nagaland	Rhizome	Pudding using rhizome powder with milk and sugar is used as general tonic for children. The powder of rhizomes with honey is applied on the mucous membrane of the oral cavity. Rhizome paste is applied to cattle injured by leech.	Demulcent, antipyretic, effective against gravel Stomatitis Aid in blood coagulation	Antifungal (Banerjee and Nigam1977)
C. aromatica	Bon haladhi (Assamese); Lam-yaingang (Manipuri)	Arunachal Pradesh, Assam, Manipur, Meghalaya	Rhizome	Paste of rhizome with milk is used for blood dysentery and stomach-ache. Paste of rhizome taken with water.	Carminative, antidote to snake bite, astringent and used for bruises, corns and sprains Kills intestinal worms	Inhibits proliferation of hepatoma (Wu et al 2000) Anticancer (Li et al 2008) Larvicidal (Madhu et al2010)
C. caesia	Kalahalud (Assamese); Amuba yaingang (Manipuri); Aihang (Mizo); Chongkah (Khampti, Arunachal Pradesh); Homen	Arunachal Pradesh, Assam, Mizoram	Rhizome and seeds	The dried rhizome powder is mixed with powdered seeds of *Andrographis paniculata* and applied	Anti inflammatory, anti-asthmatic	Antimicrobial (Garg and Jain 1998), Antifungal (Banerjee and Nigam, 1976)

	(Lohit, Arunachal Pradesh)			during insect, scorpion and snake bite. Fresh rhizome juice along with mustard oil is given daily. Rhizome paste	Dysentery For healing of wound	
C. domestica	Haldi (Assamese); Yaingang (Manipuri)	Arunachal Pradesh, Assam, Manipur, Nagaland	Rhizome	The dried powder of rhizome.	Wound healing, anti-inflammatory flatulence, jaundice, scabies	Anticancer (Shankar and Srivastava 2007), Antioxidant (Ramsewak et al2000), Anti-inflammatory (Ramsewak et al2000,Chainani-Wu,2003), Antifungal (Apisariyakul et al 1995),Antibacterial (Kim et al 2005), Antiviral (Sindelarova et al1996)
C. longa	Haladhi (Assamese); Aieng (Mizoram); Khumein Nak (Khampti, Arunachal, Pradesh)	Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Tripura	Rhizome	Crushed rhizomes are taken orally before food. Rhizomes are chewed for relief from asthma.	Dyspepsia Vasodilator	Anti-inflammatory, Antispasmodic activity (Ammon and Wahl, 1999), Anti-HIV, Antioxidant, Anti-tumour, Anti venom (Araújo and Leon 2001)
C. zedoaria	Keturi (Assamese); Aidizung (Mizo).	Arunachal Pradesh, Assam, Mizoram	Rhizome	Rhizome extract	Blood purifier, cough antiseptic, indigestion, wound healing, toothache, leucoderma, tuberculosis, enlargement of spleen and for promoting menstruation.	Anticancerous (Syu et al 1998)

K. galanga	Chandramula (Assamese); Sying khmoh, Sying shmoh (Khasi)	Arunachal Pradesh, Assam	Leaves and rhizomes	The rhizome is externally used. Rhizome is taken orally.	Treating indigestion, cold, pectoral and abdominal pains, headache, carminative and toothache, menstrual pain, insecticidal. Effective for dandruff or scabs on the head Against poisoning when there is blood vomiting	Larvicidal activity (Othman et al 2006), Inhibits activity of Epstein-Barr virus (Kanjanapothi et al 2004), kills larvae of the mosquito (Ahn et al 2008)
K. pulchra	Khanjanburah (Assamese)	Assam, Mizoram	Rhizome leaves and stem	Rhizome paste is used in the treatment of pneumonia and bronchial complaints. Steamed rhizomes, stems and leaves for curing wound.	Pneumonia, bronchial complaints Wound healing	Topical anti-inflammatory activity (Pongprayoon et al1996)
K. galanga	Chandramula (Assamese); Sying khmoh, Sying shmoh (Khasi)	Arunachal Pradesh, Assam	Leaves and rhizomes	The rhizome is externally used. Rhizome is taken orally.	Treating indigestion, cold, pectoral and abdominal pains, headache, carminative and toothache, menstrual pain, insecticidal. Effective for dandruff or scabs on the head Against poisoning when there is blood vomiting	Larvicidal activity (Othman et al 2006), Inhibits activity of Epstein-Barr virus (Kanjanapothi et al 2004), kills larvae of the mosquito (Ahn et al2008)
K. pulchra	Khanjanburah (Assamese)	Assam, Mizoram	Rhizome leaves and stem	Rhizome paste is used in the treatment of pneumonia and bronchial complaints. Steamed rhizomes, stems and leaves for curing wound.	Pneumonia, bronchial complaints Wound healing	Topical anti-inflammatory activity (Pongprayoon et al1996)

H. coccineum	Aichhia (Mizoram); Mansila (Lohit Arunacahal Pradesh)	Arunachal Pradesh, Mizoram	Rhizome	Rhizome paste applied over swollen part.	Anti-inflammatory	None
H. coronarium	Pakhila phul (Assamese); Tora (Chakma Arunachal Pradesh)	Arunachal Pradesh, Assam	Flowers, rhizome and stem	Flower paste Rhizome extract prepared by boiling the rhizome in water. Essential oil from rhizome.	Foetid nostrils Febrifuge, tonic and anti rheumatic swellings Antihelmintic, tonic and mild tranquiliser	Antibacterial (Aziz et al2009)
H. spicatum	Takhellei-hanggam-mapan (Manipuri); Aithur (Mizo) Karpurakachari (Bengali)	Arunachal Pradesh, Manipur, Meghalaya, Mizoram	Rhizome	Root decoction Powder of root	Nausea, Bronchial Asthma, Halitosis, Vomiting and indigestion, expectorant; stimulant; stomachic Treatment of liver complaint, treating fevers, vomiting, diarrhoea, inflammation, pains and snake bite, heating potency to the female, stimulant, expectorant, tonic, carmative	Antibacterial against methicillin and vancomycin resistant *Staphylococcus aureus* and fungal cultures (Bisht et al 2006)

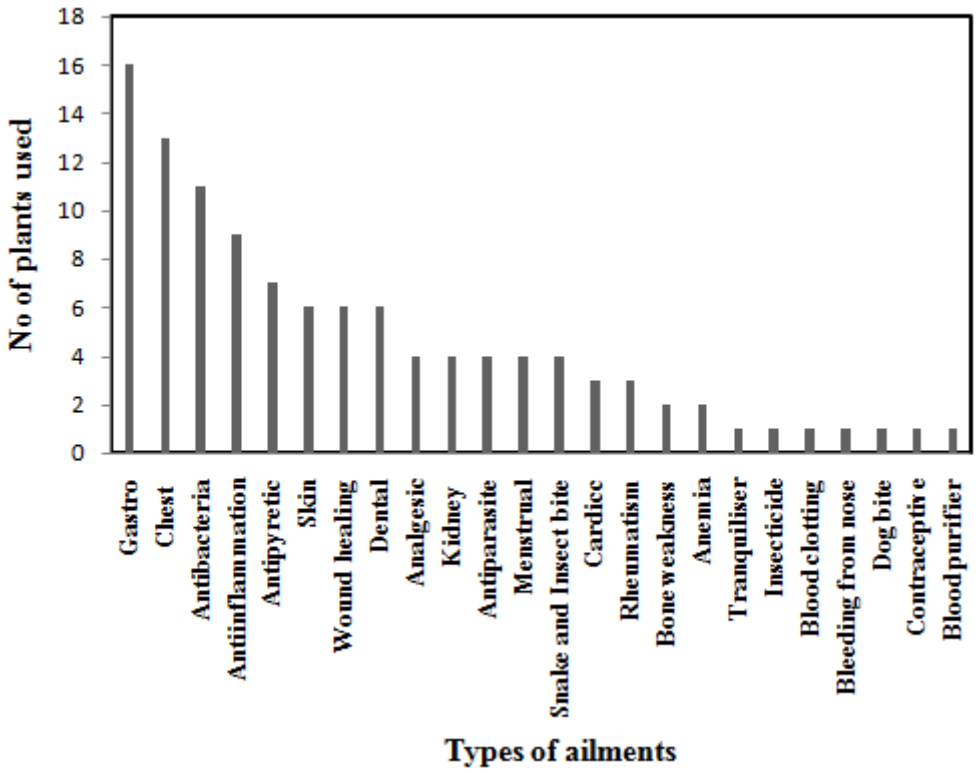

Fig 3.4 Percentage use of Zingiberaceous plants for treating various human ailments. Highest percentage of plants used is observed for treatment of gastrointestinal ailments that is also supported by large number of pharmacological reports. Percentage was calculated over number of plants documented in the current study.

3.3.5 Cytological analysis of subfamily Zingiberoideae

3.3.5.1 Collection of root tips

The best time of the day to collect root tips for cytological study was found to be between9-11 am where there are more cells during metaphase stage. For *Curcuma* and *Hedychium*, the optimum time of collection was found to be from 10 am to 10.30 am, whereas the best time for chromosome observation in *Kaempferia* was from 9 am to 9.30 am. However, the midday period is widely followed in the field of cytology for the family Zingiberaceae (Lim 1972). Midday is known to be at a peak of cell division in many plants and thus will yield the highest number of metaphases when fixed for cytological observation. Late morning period was also preferred by some workers (Chen 1992; Augsonkitt et al 2004).

3.3.5.2 Pre-treatment

Pre-treatment is a necessary step in cytological studies of members of Zingiberaceae. Different workers have used various pre-treatments and staining chemicals in their studies (Chen 1992; West and Cowley 1993; Rai et al 1997; Das et al 1999; Joseph et al 1999; Nair & Sasikumar 2009). This helps to block the mitosis cycle at metaphase stage by inhibiting spindle fibre formation revealing more number of cells in metaphase. PDB and 1-bromonapthalene (MBN) were found better in treating the Zingiberaceae plants (Chen 1992). However, in this study it was found that PDB alone was effective in treating Zingiberaceous plants than the mixture of PDB and 8-hydroxyquinolene (OQ) in terms of yielding slightly higher percentage of metaphase cells.

3.3.5.3 Hydrolysis and staining

Hydrolysis of the root tips is carried out to soften the root tissue. The roots of monocots are harder and larger in size and therefore, it is an important step to soften the pre-treated roots for a specified period in a 1:1 (v/v) mixture of 1N HCl and absolute ethanol. In the present study, the time was optimized as30 sec for all the species at room temperature and immediately squashed in a drop of 1% lacto propionic orcein. Orcein is another stain giving fruitful results in many members of Zingiberaceae (Nair & Sasikumar 2009).

3.3.5.4 Slide preparation, squash and observation

The preparation of a good slide depends on the proper handling of the root material, cutting, maceration and squashing. Care was taken so that the root tip was not damaged. One drop of lacto propionic orcein was added to the slide containing squashed root tips. The squashing and spreading of the cells uniformly is a crucial step which results in proper visualization of the slide. The slides revealed well-spread metaphases under low power (10X) and high power (40X) magnifications in compound light microscope. Some cells were found to be in anaphase and late prophase stages also.

3.3.5.5 Chromosome number determination

The basic idea of incorporating the cytological information with the morphology is due to the characteristics of a particular karyotype associated with a complex of characters circumscribing a family, tribe, genus, species, or race. When certain peculiarities such as size of chromosomes and basic number are concerned, their correspondence to higher taxonomic subdivisions does not imply that they did not derive from lower categories; it only indicates a greater phylogenetic stability of these peculiarities (Jackson 1971). Changes in chromosome morphology may lag behind or precede change in external phenotype. Close morphological similarity of taxa associated with great differences in the basic karyotype suggests that cytological differences have been by sudden fragmentations, translocations, etc. Partial correspondence of morphology and karyotype implies parallel changes. The chromosome number of the 9 species of sub family Zingiberoideae was investigated by conventional rapid squash technique. The chromosome number of each species is given in Table 3.3. The chromosome spectrum of NE Indian Zingiberoideae varied from 2n=21 to 2n=63with majority of the species concentrated in the number 2n=34. It was seen that chromosome number was found variable in the genera *Curcuma* and *Kaempferia.* However, the chromosome number showed consistency in case of *Hedychium.*

Kaempferia showed two distinct lines of evolution with 2n=22 (*K. pulchra* and *K. angustifolia*) and 55 (*K. galanga*) implicating the diploidy (2x) and pentaploidy (5x) status (Fig 3.5A-3.5B). The basic chromosome number (x) for the genus *Kaempferia* was 11. Intra species variation of chromosome number

predominates the genus. A similar trend has been observed in the previous literature for *K. pulchra* (Joseph 2010). For *K. angustifolia,* chromosome number reported in the literature to be 2n=33 (Eksomtramage et al 2001) but current report of 2n=22 deviate from the previous report. For *K. galanga,* somatic chromosome number was found to be 2n=54 (Joseph 2010). However the current finding of somatic chromosome count of *K. galanga* suggest that 2n=55 deviates from the previous study. Chromosome reports available on *Kaempferia* show that a wide range of diploid chromosome numbers ranging from 2n=22-54 occurs in the genus. The data indicate that the species of the genus fall under several basic series such as x=9, 11, 12, 13 and 14 of which x=11 is the most frequent one. It may be noted that species of *Kaempferia*, on morphological considerations, fall under apparently two types such as those showing morphological specialities characterized by the two lateral petaloid staminodes free from the deeply two-lobed labellum, and others with fused labellum and staminodes (Omanakumari & Mathew 1984).The former morphologically less advanced species are Asiatic, and the latter advanced ones from African (Mahanty 1970)

The occurrence of different ploidy levels in *Curcuma* was highlighted in early cytological studies (Chakravorty 1948; Sharma & Bhattacharya 1959). Three different chromosome counts were found for *Curcuma* 2n = 21(*C. caesia*), 42 (*C. longa*) and 63 (*C. zedoaria*) implicating the triploid, hexaploid and nonaploid status of the studied Zingiberaceae species with the basic chromosome number of x=7 (Fig 3.5C-3.5D). The majority of Indian *Curcuma* is reported to possess the basic chromosome number x=7 and published count corresponds to 6x, 9x, 11x, 12x and 15x ploidy levels (Shornickova et al 2007). The reported chromosome numbers of *C. caesia* is found to be 22 (Das et al 1999), 63 (Islam 2004, Joseph et al 1999).*C. longa* locally named as Halodhi, the published chromosome numbers were found to be 2n=32 (Sato et al 1948), 2n=48 (Das et al 1999), 2n=62, 63 and 64 (Chakraborty et al 1948). Our report (*C. longa* 2n=42) is in close agreement with the chromosome number studied earlier (Das et al 1999).*C. zedoaria* locally named as keturi, the published chromosome number were 2n =63, 64, 66 (Prana 1977). Our report (*C. zedoaria* 2n=63) is also in the agreement of the previous published count (Prana 1977).

Although the inter species variation in chromosome number is very well studied (Joseph 2010; Fei et al 2010) ranging from 2n=34 to 2n=68, *H. coronurium* showed both diploid and triploid numbers with 2n=34 and 2n=51(Joseph 2010). *H. flavescens* showed 2n=51. For the three species of *Hedychium* the 2n chromosome numbers were found to be 34 (*H. coronarium*, *H. chrysoleucum* and *H. gardnerianum*). The species are natural diploid with the basic chromosome number of x = 17 (Fig 3.5E-3.5G).

3.3.5.6 Karyomorphometrical analysis

Since, most of the members of Zingiberoideae possess very small chromosomes; the detailed karyomorphological study by conventional methods was not easy. The general feature noted in the sub family Zingiberoideae was the wide range of chromosome with very small sized in most of the species. However, the chromosome complements in the various members differ in minute karyotypical details (Table 3.3). The chromosome range from 0.30-2.32 µm in length (0.24 to 2.68 µm, Joseph 2010). The average chromosome length (ACL) varies from 0.53-1.44 µm (0.39 to 1.69 µm, Joseph 2010). The total chromosome length (TCL) shows a very wide variation with 15.06 µm being the minimum and 79.22 µm being the maximum value (TCL_{Min}= 16.21 µm, TCL_{Max}= 88.56 µm, Joseph 2010). The disparity index value ranges between 30.00 to 56.52 (27.9 to 64.7, Joseph, 2010). The coefficient of variation ranges from 19.05 to 32.90 (12.76 to 41.27, Joseph 2010).

The high value of average chromosome length shown by *H. chrysoleucum*, *K. pulchra* and *K. galanga*(Table 3.3, Fig 3.6A) probably show their primitiveness, whereas the lower values for these parameters found in *H. gardnerianum* and *K. angustifolia* denotes their evolved nature. A decrease in chromatin length is one of the factors responsible for evolution of higher plants (Babcock & Cameron 1934). The comparatively high disparity index (DI) value found in *H. chrysoleucum*, *C. zedoaria* and *K. galanga* corresponds to the heterogeneous assemblage of chromosomes in these taxa (Table 3.3, Fig 3.6B).

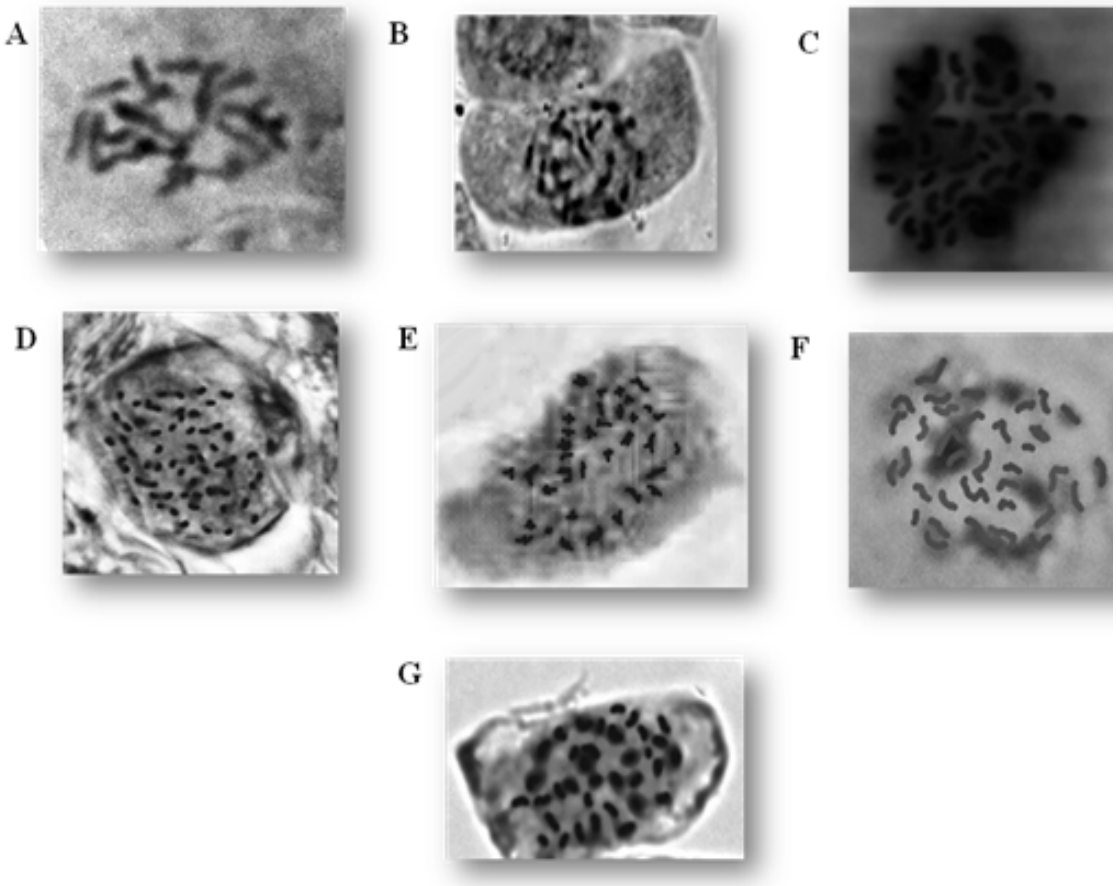

Fig 3.5 Mitotic chromosomes (2n) from root tips of 7 Northeast Indian Zingiberaceous species. *K. pulchra* (2n=22) (A), *K. angustifolia* (2n=22) (B), *C. longa* (2n =42) (C), *C. zedoaria* (2n=63) (D); *H. chrysoleucum* (2n=34) (E); *H. coronarium* (2n=34) (F), *H. gardnerianum* (G) (2n=34).

Table 3.3 Summary of the karyomorphometrical analysis on the nine members of Zingiberoideae investigated

		In this study							Literature report (Joseph 2010)						
S. No	**Species**	**Chromosome number (2n)**	**Karyotype formula**	**Total chromosome length in µm**	**Range of chromosome length in µm**	**Average chromosome length in µm**	**Disparity index**	**Variance coefficient**	**Chromosome number (2n)**	**Karyotype formula**	**Total chromosome length in µm**	**Range of chromosome length in µm**	**Average chromosome length in µm**	**Disparity index**	**Variance coefficient**
1	*C. caesia*	21	A1B18C2	15.06	0.42-1.05	0.68	42.86	24.18	63	A4B28C31	32.33	0.34-0.89	0.51	45.50	21.82
2	*C. longa*	42	B36C6	27.51	0.39-0.99	0.665	43.47	24.40	53	A2B20C41	31.97	0.40-1.01	0.51	45.00	20.68
3	*C. zedoaria*	63	B24C39	20.52	0.38-0.83	0.60	55.22	20.79	63	A4B4C55	27.23	0.31-0.66	0.43	36.50	17.24
4	*H. chrysoleucum*	34	A17B17	37.61	0.55-1.98	1.11	56.52	32.90	-	-	-	-	-	-	-
5	*H. coronarium*	34	B29C4	20.52	0.35-0.83	0.60	40.68	32.90	34	A2B20C12	16.53	0.42-1.34	0.49	37.6	20.66
6	*H. gardnerianum*	34	B20C14	17.89	0.30-0.84	0.53	47.37	22.15	-	-	-	-	-	-	-
7	*K. angustifolia*	22	A7B15	19.91	0.54-0.91	0.54	45.86	27.12		-	-	-	-	-	-
8	*K. pulchra*	22	A15B7	23.55	0.84-1.56	1.17	30.00	19.05	22	A6B16	22.71	0.79-1.25	1.03	27.9	12.76
9	*K. galanga*	55	A51B4	79.22	0.77-2.32	1.44	50.11	23.79	54	A14B40	86.55	0.99-2.68	1.64	48.30	22.79
	Range	22-63		15.06-79.22	0.30-2.32	0.53-1.44	30.00-56.52	19.05-32.90	34-63		16.53-86.55	0.31-2.68	0.49-1.64	36.50-48.30	17.24-21.82

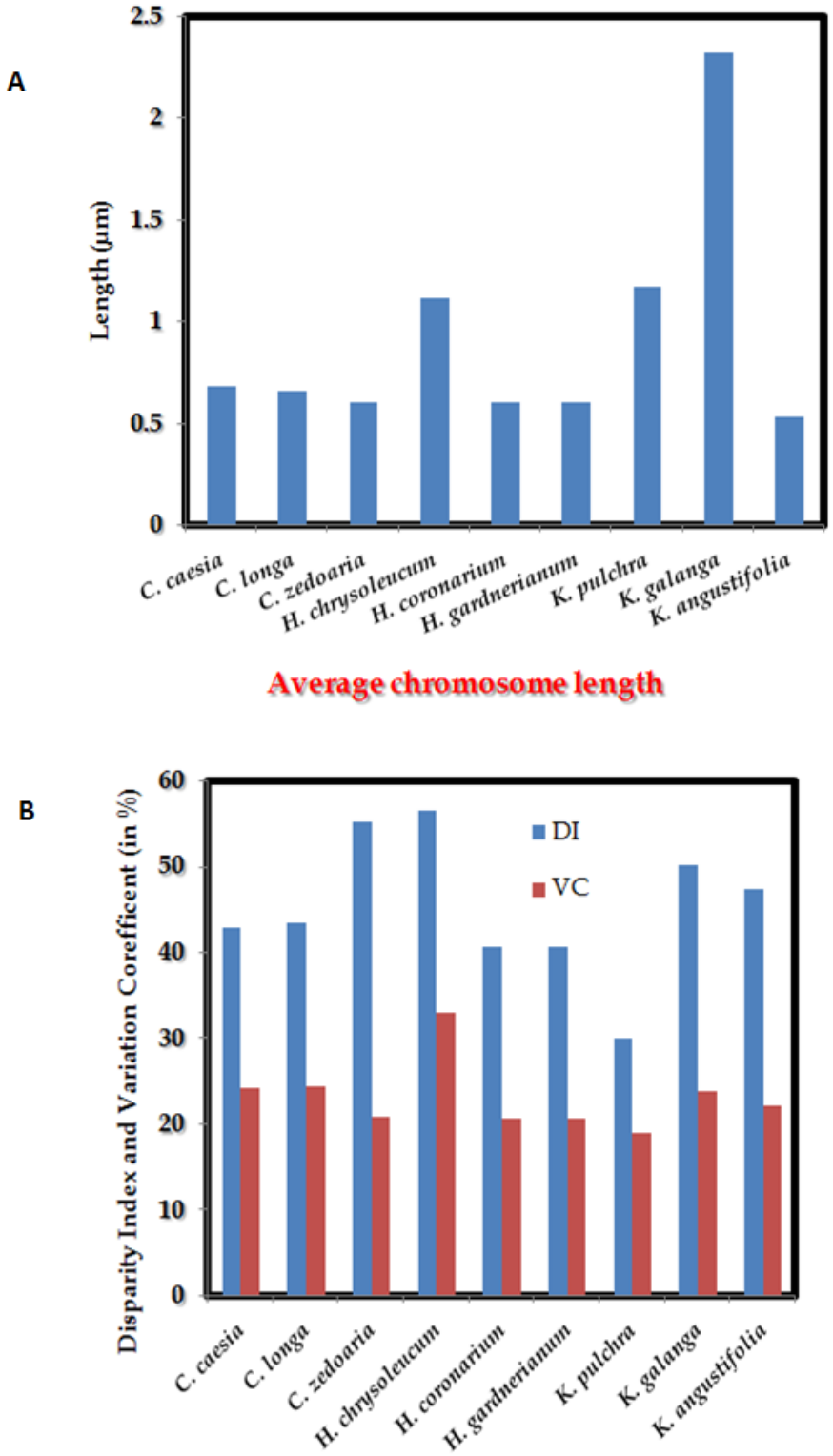

Fig 3.6 Comparison of major karyotypic parameters in nine species of Zingiberoideae. **A.** Average chromosome length B**.** Disparity index and variation coefficient.

But comparatively lower values of DI found in other members (*C. caesia, C. longa, H. coronarium, H. gardnerianum, K. angustifolia* and *K. pulchra*) point towards the general homogeneity found in various species of sub family Zingiberoideae. Normally a low disparity index value corresponds to the homogeneity of chromosomes in most of the higher as well as lowers plants (Mohanty et al1991).

3.4 Conclusion

Given the diversity of sub family Zingiberoideae in NE India, it becomes imperative that efforts are made to preserve the wild genotypes of Zingiberoideae of this area. From this study of Assam, Mizoram, Manipur itself, it has yielded two rare and endemic species giving an estimation of the high species richness of the region. The subjective evaluation gives an idea of the most potential species which can be sustainably utilized. But more studies on its molecular and biochemical aspect need to be conducted to cement this evaluation. The present study represents work of limited nature but draws attention to the rich bio-resources which forms a foundation for further scientific research on medicinal plants significant to both developing and developed countries. Considering the rapid loss of biodiversity, a need is felt to document the medicinal uses of various plants and their conservation too. North East India is not only an important reservoir of rich flora but the region also comprises diverse ethnic population and their traditionally conserved knowledge. In this study an attempt was made to cover use of Zingiberaceae family by aboriginal people of NE India. A total of 14 plant species belonging to the sub family Zingiberoideae were identified to possess medicinal importance and primary activity seems to be against gastrointestinal ailments. Most of the persons from whom we had gathered the information are illiterate and at times there is no script for the language they speak, hence the herbal practices are running in mouth to mouth without any written documents. Therefore there is an utmost need to document the traditional and cultural practices including from remotely located region where in the tribes and communities has been living in harmony for century's altogether.

The work has brought to light some hidden but popular prescriptions of an ethnic group. A concrete list of plants and their utilization will not only provide basic data for further studies aimed at conservation, cultivation, healthcare and economic welfare of rural and tribal people but all future pharmacological and clinical studies. Since NE region is rich in species of Zingibereceae, the extensive ethnobotancial study should be carried out. This report is the good example which compiles the ethanobotanical information from sub family Zingiberoideae that may lead to the discovery of potentially valuable pharmaceuticals.

From the cytological studies of the 9 investigated Zingiberoideae germplasm, it can be concluded that speciation and evolution have been possible as a result of increase in the variability through changes in the base numbers, as well as numerical and structural changes in the chromosome number. The diverse cellular phenomenon, for example, autopolyploids, allopolyploids, protoautoploidy, amphiploidy, ascending and descending dysploidy might have resulted in the variability of the base numbers in the family. The wide range of chromosome numbers observed in many genera in the present study mark a significant contribution that aneuploidy and polyploidy have played in the evolution of various taxa at the generic and species level. It also appeared that various kinds of aberrations have played key role in the evolutionary diversification of the family. Individuals with same chromosome number but with difference in karyomorphological details reflect the ongoing evolutionary process at micro level.

CHAPTER 4

GENETIC DIVERSITY AND RELATIONSHIP IN ZINGIBEROIDEAE

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This chapter gives the genetic diversity and relationship of Zingiberoideae species.

CHAPTER 4A

EVALUATION OF GENETIC DIVERSITY IN TURMERIC (*CURCUMA LONGA* L.)

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This chapter discusses the genetic diversity status of the turmeric cultivated varieties of different states of Northeast India.

EVALUATION OF GENETIC DIVERSITY IN TURMERIC (*CURCUMA LONGA* L.)

4A.1 Introduction

Turmeric (*C. longa* L. syn. *C. domestica* Val.), family Zingiberaceae, is a pan tropical crop cultivated widely in South East Asia. Due to its multitude use as spice, natural dye, food preservative and therapeutic agent, it is gaining high demand in food, cosmetic and pharmaceutical industries. Turmeric is considered to be a triploid [2n = 3x = 63; x = 21] (Islam 2004), though a recent report based on the flow cytometric data and chromosome counts suggested a new ploidy status [9x] by defining a new basic chromosome number of x = 7, without contradicting the triploid status (Skornickova et al 2007). Although turmeric is propagated clonally, viable sexual reproduction is also reported (Sasikumar et al 1996).

The decreased import and increased export of turmeric has contributed to the growth and economy of India. India is a leading producer and exporter of turmeric in the world. Andhra Pradesh, Tamil Nadu, Orissa, Karnataka, West Bengal, Gujarat, Meghalaya, Maharashtra, Assam are some of the important states growing turmeric on commercial basis, of which, Andhra Pradesh has the highest productivity. Although the market price for other spices (onion, black pepper, large cardamom, coriander, ginger, clove etc.) is increasing by leaps and bounds, the market price of turmeric has become quite stable in the market. Also, the productivity of the crop is much lower (1.87t/ha) in NE states compared to the national productivity which is 3.47 t/ha (Spices Statistics, Spices Board, 2004). This is not only due to the acidic nature of soil (due to excessive rainfall), jhum cultivation, but also different kinds of diseases like leaf blotch (*Taphrina maculans*), leaf spot (*Colletotrichum capsici*), rhizome rot (*Pythium graminicolum*), and root knot nematodes (*Radopholus similis*) spoiling the productivity of turmeric. Molecular markers employed for characterization studies of turmeric are limited to the application of RAPD (Salvi et al

2001, Panda et al 2007; Tyagi et al 2007), RAPD and or ISSR markers (Hussain et al 2008), isozyme markers and SSR markers (Sigrist et al 2010). There exists no report on the genetic diversity assessment of NE turmeric varieties. So an urgent need has been felt to assess the genetic diversity of cultivated turmeric varieties status of turmeric of different states of NE India. Current study was therefore undertaken to understand the genetic diversity status of four states (Assam, Manipur, Arunachal Pradesh and Mizoram) of NE India.

4A.2 Materials and methods

4A.2 .1 Plant materials

The present investigation deals with 19 local cultivated varieties of *C. longa* collected from four different states of NE India (Table 4A.1; Fig 4A.1). The rhizomes collected in duplicates were grown in the greenhouse of the Department of Biotechnology, IIT Guwahati, Assam. Geographical position of the collection sites are shown in Fig. 4A.1. Herbarium vouchers are deposited in the herbarium Gauhati University Botanical Herbarium (GUBH, recognized by American Botanical Society), with duplicates maintained at IIT Guwahati, Assam.

4A.2 .2 DNA extractions

Total genomic DNA was extracted from fresh tender leaves of the material using a DNeasy Plant mini kit (Qiagen Inc., Venlo, Netherlands). The quality and quantity of the extracted DNA was confirmed to be consistent by running the extracted DNA on 0.8 % agarose gel, stained with ethidium bromide (0.5 µg μL^{-1}).

Table 4A.1 List of *C. longa* cultivated varieties used in this study

S. No	*C. longa* variety (Accession No)	State of Origin
1	12951	Assam
2	12953	Assam
3	12955	Assam
4	12950	Assam
5	12956	Assam
6	12951	Arunachal Pradesh
7	12940	Arunachal Pradesh
8	12949	Arunachal Pradesh
9	12947	Arunachal Pradesh
10	12931	Arunachal Pradesh
11	12956	Manipur
12	12978	Manipur
13	12989	Manipur
14	12939	Manipur
15	12910	Manipur
16	12980	Meghalaya
17	12944	Meghalaya
18	12990	Meghalaya
19	12985	Meghalaya

4A.2.3 RAPD and ISSR analysis

PCR amplification of the genomic DNA was carried out using 20 RAPD and 20 ISSR (Operon Tech, USA) primers to study the intra-varietal and inter-varietal genetic relationship (Table 4A.2). The reaction mixture of 12.5 µL contained 50 ng μL^{-1} of template DNA, 1X Readymix (Bioline), 3.0 mM $MgCl_2$, (Bioline), 5 pM of each primer. The reaction was performed in 0.2 mL microfuge tubes (Axygen). PCR amplification was carried out in a Mini Thermal Cycler (Takara). Thermal cycling conditions were as follows: pre-denaturing step of 5 min at 94 ^oC, followed by 35 cycles each of 45 s at 94 ^oC, annealing for 1 min at 32 ^oC, extension for 1 min at 72 ^oC, and followed by one final extension cycle of 10 min at 72 ^oC. The annealing temperature for 1 min varied from 38 - 50 ^oC for ISSR primers depending on the melting temperature of the primer (Table 4A.2). The amplification products were electrophoresed in 1.5% agarose gels in 1X TAE (stock contained 2.0 M Tris, 0.8 M Acetic acid and 0.5 M EDTA). The gels were photographed under a UV light with the help of gel documentation system (BioRad, India).

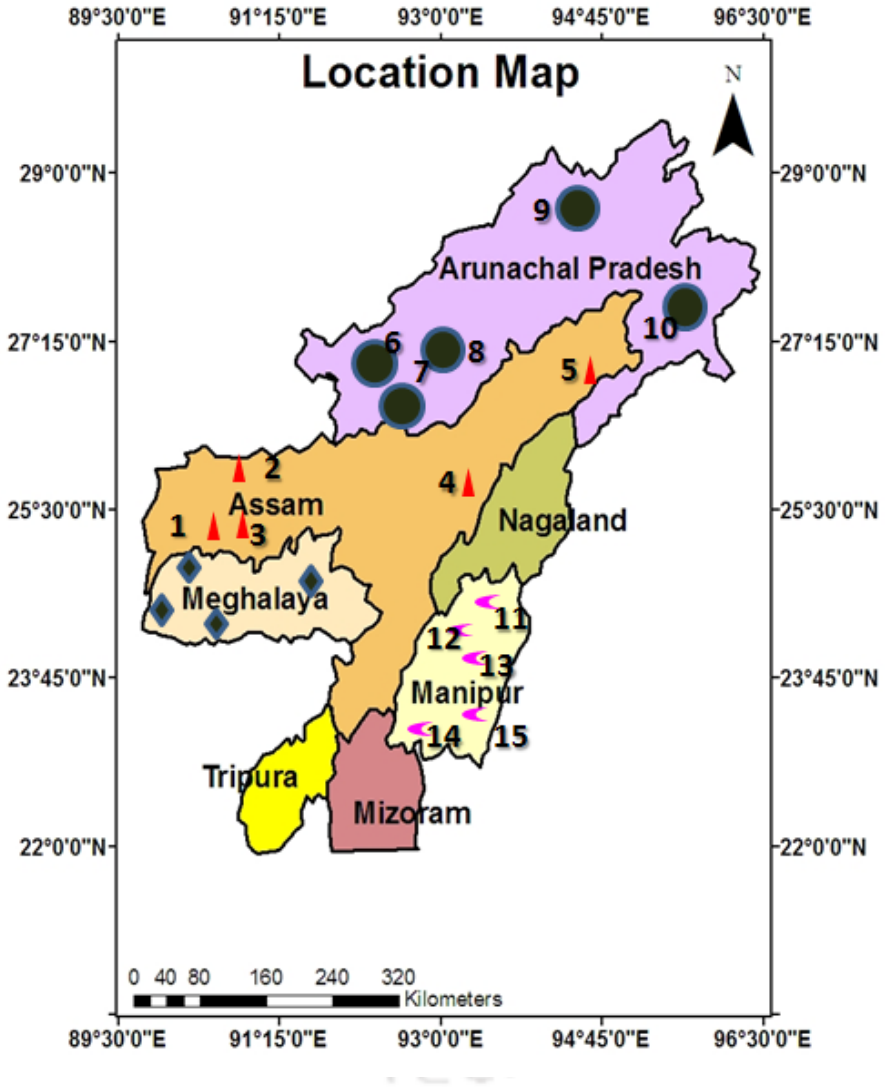

Fig 4A.1 Geographical distribution of the *C. longa*. Numbering correspond to the code specified in the table 4A.1

Table 4A.2 Sequence information of RAPD and ISSR oligonucleotide primers

S. No.	RAPD Primer	Sequence (5´-3´)	Annealing temp (°C)	S. No.	ISSR Primer	Sequence (5´-3´)	Annealing temp (°C)
1	OPA01	CAGGCCTTC	32	1	17899A	CACACACACACAAG	40
2	OPA 02	ACGATGAGCC	32	2	17899B	CACACACACACAAC	43.7
3	OPA 03	AGTCAGCCAC	32	3	17898A	CACACACACACAAC	40
4	OPA 04	AATCGGGCTG	32	4	17898B	CACACACACACAAG	39
5	OPA 05	AGGGGTCTTG	32	5	807	AGAGAGAGAGAGAGAGT	42
6	OPA 06	GGTCCCTGAC	32	6	844	CTCTCTCTCTCTCTCTAC	43.3
7	OPA 07	GAAACGGGTG	32	7	811	GAGAGAGAGAGAGAGAC	43
8	OPA 08	GTGACGTAGG	32	8	814	CTCTCTCTCTCTCTCTTG	44.7
9	OPA 09	GGGTAACGCC	32	9	824	TCTCTCTCTCTCTCTCG	47
10	OPA 10	GTGATCGCAG	32	10	825	ACACACACACACACACT	46.2
11	OPA11	CAATCGCCGT	32	11	826	ACACACACACACACACC	49.6
12	OPA12	TCGGCGATAG	32	12	HB12	CACCACCACGC	42.4
13	OPA 13	CAGCACCCAC	32	13	HB13	GAGGAGGAGGC	38
14	OPA14	AAGTGCGACC	32	14	HB14	CTCCTCCTCGC	41
15	OPA 15	TTCCGAACCC	32	15	HB15	GTGGTGGTGGC	42
16	OPA16	AGCCAGCGAA	32	16	809	AGAGAGAGAGAGAGAGG	46.6
17	OPA17	GACCGCTTGT	32	17	816	CACACACACACACACAT	46.6
18	OPA18	AGGTGACCGT	32	18	817	CACACACACACACACAA	46.6
19	OPA19	CAAACGTCGG	32	19	872	GATAGATAGATAGATA	34.7
20	OPA 20	CAGCCGAGAA	32	20	SSRU	CAGCAGCAGCAGCAG	40.0

4A.2 .4 Data analysis

For scoring and analysis of data from two molecular markers, sample was tested in triplicates. The total numbers of monomorphic and polymorphic bands which were clear, unambiguous and reproducible were scored for all tested primers. Scoring of data was carried out using binary number system for the presence or absence of each fragment in each sample. To avoid taxonomic ambiguities, all bands were taken into considerations, only the presence of band was taken as an indicative. Polymorphic information content (PIC) was used to compare the efficiency of each primer, computed using the formula PIC = $1 - \sum pi^2$, where pi is the frequency of i^{th} allele at a given locus (Anderson et al 1993) and also marker index (MI) was calculated (Powell et al 1996). Diversity was estimated at different level using POPGENE, Version 1.32 (Yeh et al 1997). Unbiased estimates of Nei's genetic identity (*I*) and genetic distance (*GD*) (Nei 1972) were calculated. The levels of genetic variability within variety were estimated using variables like, the observed number of alleles per locus (N_a), the effective number of alleles per locus (N_e), proportion of polymorphic loci (*P*) per variety, Shannon's information index (I), Nei's gene diversity (h). For each polymorphic locus, total genetic diversity (H_T), which was partitioned into diversity within varieties (H_S) and diversity among varieties (and D_{ST}) as $H_T = H_S + D_{ST}$ was measured. A measure of genetic differentiation among varieties relative to the total genetic diversity (G_{ST}) was calculated at each polymorphic locus ($G_{ST} = D_{ST} / H_T$). A rough estimation of the quantity N_m (*N* = size of variety, m = migration rate) was also estimated using formula $F_{ST} = 1 / (1+4Nm)$ (Wright 1951). Percentage of polymorphic bands and matrix of genetic similarity compiled using Dice's coefficient was used as a measure of degree of similarity. A dendrogram representing the genetic relationship among the varieties was generated using UPGMA clustering applying the SHAN subroutine through the NTSYS-pc (Numerical taxonomy system, 2.2 versions: Numerical taxonomy system, Applied Biostatistics, NY) (Sneath & Sokal 1973). To know the goodness of fit to a specific cluster in the UPGMA similarity matrix, the relation between the original similarity matrices and cophenetic values were evaluated, and the Mantel's test was executed for it. Principal component analysis (PCA) was also performed for the families with modules STAND, CORR, and EIGEN of NTSYS-pc using the Euclidean distances derived from the standardized

values using the NTSYS-pc-2.2. Gene flow (Nm) was estimated from Nm = 0.25 × (1 − Gst) / Gst. Analysis of molecular variance (AMOVA) was performed using Arlequin version 3.01 (Excoffier et al 2005) at two hierarchical levels to examine differences among and within varieties. The Fixation index or F statistics (F_{ST}) was also calculated with Arlequin v. 3.01. The significance of this analogy was evaluated by 1000 random permutation of sequences among varieties.

4A.3 Results and discussion

4A.3.1 Marker analysis (RAPD and ISSR)

For analysis of genetic diversity of turmeric germplasm, 20 RAPD and 20 ISSR primers were used. Of 20 RAPD primers, 9 gave successful amplification with a total of 51 bands, of which 50 were polymorphic (98.0 %) with the average PIC and MI of 0.34 and 33.66 respectively. The most informative RAPD primer was found to be OPA 18 (PIC = 0.43). The percentage of polymorphic fragments ranged from 83.3% to 100.0 %. Apart from OPA10, all RAPD primers exhibited the highest polymorphism percentage. The amplification products was 5.66 per primer, the maximum was 12 with OPA11, whereas the minimum was 2 with OPA19. Successful amplification was observed for 8 ISSR primers producing 30 amplification products of which 25 (83.3 %) were polymorphic with the average PIC and MI of 0.22 and 19.57 respectively. The primers HB13 and 811 exhibited the maximum polymorphism percentage for ISSR analysis. The amplification products was 3.75 per primer, the maximum was 7 with 811, whereas the minimum was 2 with HB12 and 818. The genetic distance recorded using DICE coefficients of similarity ranged from 0.36 to 0.93 (RAPD) and 0.65 to 0.98 (ISSR) (Table 4A.3). Amplification of huge number of polymorphic fragments illustrated that the primer sets used could be of significance for the assessment of genetic diversity in turmeric cultivars.

Jan et al (2011) showed that 96.84% of the bands were polymorphic between 20 genotypes of turmeric (*C. longa* L.) collected from three different populations of Pakistan. The work of Islam et al (2007) too supports our results that reported a high level of genetic diversity within *C. zedoaria* populations. Paisooksantivatana et al (2001) investigated the genetic diversity of *C. alismatifolia* Gagnep in Thailand using

allozyme polymorphism and found high levels of genetic diversity within a population. Furthermore, the above result was relatively very high when compared with reports relating to other RAPD findings such as *Alternaria* species, celery and wheat. The main cause for the high level of polymorphism could be intra-specific variation as reported by Nayak et al (2006) who demonstrated that high number of polymorphic loci among the turmeric cultivars.

Table 4A.3 Polymorphism detected with RAPD and ISSR primers

Primer	Sequence	PCR bands Scored	Polymorphic DNA fragments	Polymorphic DNA Fragment %	PIC	MI	Distance range (DICE coefficient)
			RAPD				0.36-0.93
OPA11	CAATCGCCGT	12	12	100.0	0.35	35.0	
OPA12	TCGGCGATAG	5	5	100.0	0.359	35.9	
OPA14	TCTGTGCTGG	7	7	100.0	0.275	27.5	
OPA16	AGCCAGCGAA	6	6	100.0	0.37	37.0	
OPA18	AGGTGACCGT	3	3	100.0	0.43	43.0	
OPA19	CAAACGTCGG	2	2	100.0	0.38	38.0	
OPA20	GTTGCGATCC	4	4	100.0	0.40	40.0	
OPA10	GTGATCGCAG	6	5	83.3	0.26	21.5	
OPA13	CAGCACCCAC	6	6	100.0	0.25	25.0	
Total/ mean		51	50	98.0	0.34	33.66	
			ISSR				0.65-0.98
17898B	$(CA)_6GT$	7	6	85.7	0.36	31.2	
HB12	$(CAC)_6GT$	2	0	0.0	0	0	
818	$(CA)_8G$	2	0	0.0	0	0	
809	$(AG)_8G$	5	4	80.0	0.15	12.1	
816	$(CA)_8T$	3	2	66.7	0.30	19.9	
HB13	$(GAG)_3GC$	6	6	100.0	0.36	36.9	
811	$(GA)_8C$	7	7	100	0.37	36.9	
Total/mean		30	25	83.3	0.22	19.57	

4A.3.2 Genetic diversity index

The observed number of alleles (Na) and effective number of alleles (Ne) ranged between 1.22 – 2.00 and 1.18 – 1.47 respectively for the overall marker system. Similarly, Nei's gene diversity (h) and Shannon's Information index (I) ranged between 0.09 – 0.28 with overall diversity of 0.28 and 0.13 – 0.24 with an average value of 0.43 respectively. The percentage of polymorphic loci (% P) was estimated in the range of 21.88% to 64.71% with an average of 54 %. Polymorphic percentage amongst 10 different Indian agro climatic populations was observed to be in the range of 51.06% to 72.34%, with an average value of 75.53% by Singh et al (2012). The

gene flow value and the diversity among variety were found to be 1.18 and 0.29, respectively (Table 4A.3). The Fixation index or F statistics (F_{ST}) was found to be 0.25. AMOVA (P <0.001) of RAPD data showed that 25% of the total genetic variability can be accounted for the differences among the turmeric varieties of 4 different states of NE India. The remaining 75% variations are due to variations among individuals within each state (Table 4A.5). The F statistics allow analysis of structures of subdivided populations. It may also be used to measure the genetic distance among subpopulations, a concept that is based on the idea that those subpopulations that are not inter-hybridising will have different allele frequencies to those of the total population (Wright 1950). High values of F statistics (Wright) (F_{ST} = 0.25) indicate large amount of genetic differentiation preserved in the turmeric varieties of NE India.

In present study, the highest no of effective alleles (Na = 1.50 ± 0.38) is observed which reveals relatively high genetic variation among the turmeric varieties. The overall genetic diversity among all varieties of *C. longa* was high possibly due to a wide range of ecological conditions within the distribution area of the turmeric varieties. In the current study, significant polymorphism (among turmeric varieties of all states ~ 76.8%) was observed. Similar dimension of variability was observed among the 39 turmeric accessions of different states of Brazil [microsatellite markers] (Sigrist et al 2011). Total genetic variability of 42% was observed among the accessions of 10 agro-climate zone of Orissa and 58% variations among individuals within the regions [RAPD and ISSR markers] (Singh et al 2012).

Table 4A.4 Genetic diversity parameters estimated by RAPD and ISSR primers for turmeric variety

Variety	Sample size (n)	Observed no of alleles (Na)	Effective no of alleles (Ne)	Nei's gene diversity (h)	Shannon's Information Index (I)	% P	H_T	G_{ST}	Nm (G_{ST})
				RAPD					
Jorhat local	5	1.65± 0.48	1.38 ± 0.37	0.22 ± 0.19	0.34 ± 0.28	64.71	0.22± 0.04		
Arunachal local	5	1.57 ± 0.50	1.39 ± 0.39	0.22 ± 0.21	0.33 ± 0.30	56.86	0.22± 0.04		
Manipur local	5	1.47 ± 0.50	1.24 ± 0.34	0.15 ± 0.18	0.23 ± 0.27	47.06	0.14± 0.03		
Meghalaya local	4	1.56 ± 0.50	1.37 ± 0.39	0.21 ± 0.21	0.32 ± 0.29	56.86	0.22± 0.04		
Inter population	20	2.00 ± 0.00	1.46 ± 0.31	0.28 ± 0.15	0.44 ± 0.18	98.10	0.28 ± 0.02	0.29	1.18
				ISSR					
Jorhat local	5	1.59 ± 0.49	1.44 ± 0.41	0.25 ± 0.22	0.36 ± 0.31	59.38	0.25 ± 0.05		
Arunachal local	5	1.22 ± 0.42	1.18 ± 0.37	0.09 ± 0.19	0.13 ± 0.27	21.88	0.09 ± 0.04		
Manipur local	5	1.50 ± 0.50	1.32 ± 0.39	0.18 ± 0.20	0.27 ± 0.29	50.00	0.18 ± 0.04		
Meghalaya local	4	1.50 ± 0.51	1.30 ± 0.36	0.18 ± 0.20	0.27 ± 0.29	50.00	0.18 ± 0.04		
Inter population	20	1.75 ± 0.44	1.50 ± 0.38	0.28 ± 0.20	0.41 ± 0.28	83.3		0.35	0.91
				RAPD + ISSR					
Jorhat local	5	1.62 ± 0.49	1.40 ± 0.39	0.23 ± 0.20	0.34 ± 0.28	62.65	0.23 ± 0.04		
Arunachal local	5	1.43 ± 0.50	1.31 ± 0.40	0.17 ± 0.21	0.25 ± 0.30	43.37	0.17 ± 0.04		
Manipur local	5	1.48 ± 0.50	1.27 ± 0.36	0.16 ±0.19	0.24 ±0.27	48.19	0.16 ±0.03		
Meghalaya local	4	1.54 ±0.50	1.35 ± 0.37	0.20 ±0.20	0.30 ±0.29	54.22	0.20 ± 0.04		
Inter population	20	1.90 ± 0.30	1.47 ± 0.34	0.28 ± 0.17	0.43 ± 0.22	92.5	0.19 ± 0.03	0.31	1.08
Range		1.22 – 2.00	1.18 – 1.47	0.09 – 0.28	0.13 – 0.44	21.88 -64.0	0.09 – 0.28	0.29 – 0.35	0.91 – 1.18

% P = percentage of polymorphic loci; H_T = variability within variety; Gst = diversity among variety; Nm = gene flow 0.25 (1 − Gst) / Gst

Table 4A.5 Analysis of molecular variance (AMOVA) for *Curcuma* varieties

	Source of Variation	d.f.	Sum of square	Variance component	Percentage of variation
RAPD	Among variety	3	54.971	2.384	25.33
	Within variety	15	105.450	7.030	74.67
	Total	18	160.421	9.414	
			Fixation Index FST : 0.253		
ISSR	Among variety	3	28.982	1.284	26.42
	Within variety	15	53.650	3.576	73.58
	Total	18	82.632	4.861	
			Fixation Index FST : 0.264		
RAPD + ISSR	Among variety	3	85.953	3.668	25.70
	Within variety	15	159.100	10.606	74.30
	Total	18	243.053		
			Fixation Index FST : 0.256		

d.f. = degree of freedom

4A.3.3 Cluster analysis

4A.3.3.1 *Inter-varietal*

The UPGMA dendrogram based on DICE similarity coefficient separated 19 varieties into two major clusters for RAPD, ISSR and combined RAPD-ISSR data sets (Table 4A.6, Fig 4A.2). Pair-wise genetic similarities with regard to RAPD, ISSR, and RAPD-ISSR markers were in the range of 0.258- 0.933, 0.541–0.979, and 0.471–0.917, with mean of 0.675, 0.438, and 0.446 respectively. The clustering pattern obtained with two types of marker data showed almost mutually independent results. However some common groupings were observed: 12 and 13 (originating from Manipur) were grouped together in RAPD, ISSR and RAPD-ISSR based dendrograms. The dendrograms produced one large cluster (Group I) and one small cluster (Group II). Group I was further divided into sub-clusters (1A-1K). The positioning of each variety according to their state of origin (for individual and combined marker system) is shown in Table 4A.7. No significant population wise segregation was observed in cluster analysis.

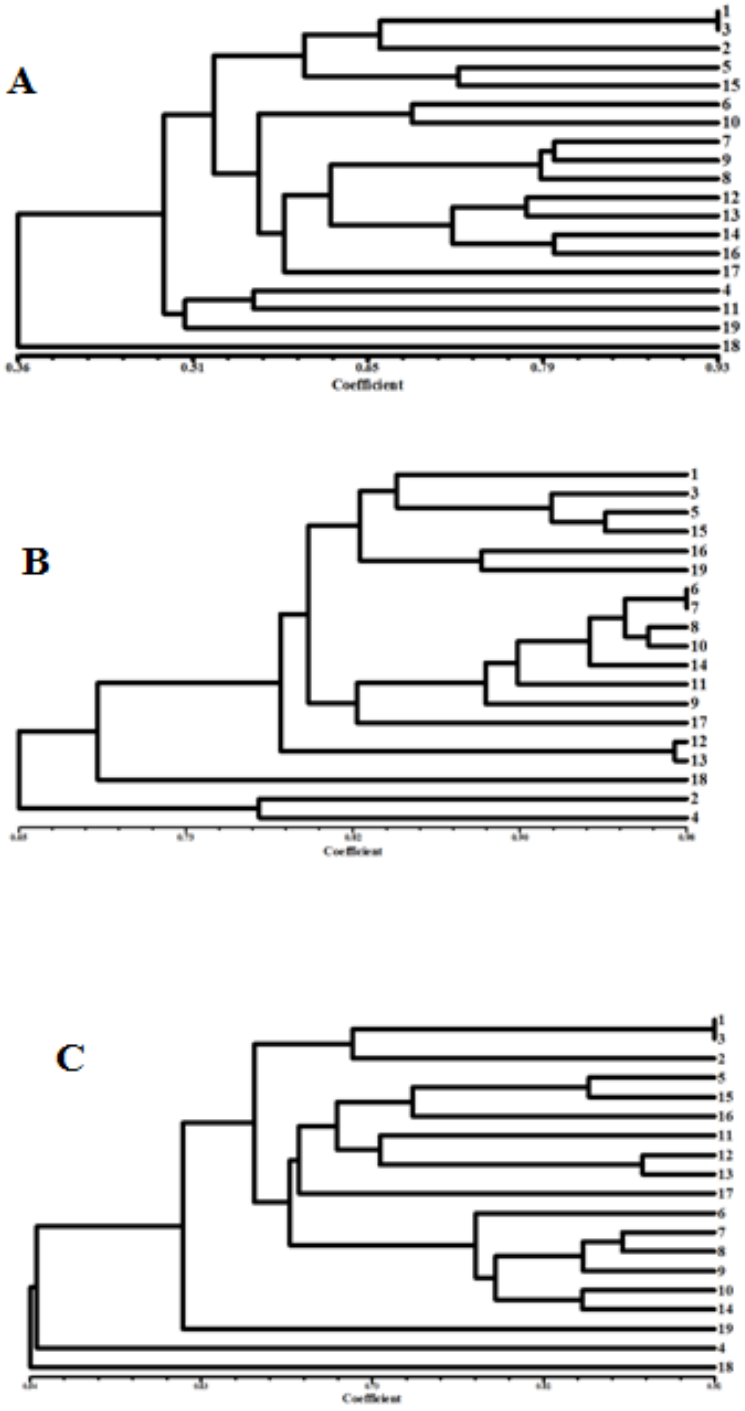

Fig. 4A.2 UPGMA dendrogram of the genetic relationships among *C. longa* varieties constructed from estimated DICE genetic similarity based on molecular markers. **A.** RAPD markers, **B.** ISSR markers and **C** RAPD-ISSR marker combinations.

Table 4A.6 Inter-varietal groupings of the *C. longa* as depicted by UPGMA dendrogram

Clusters	Sub-clusters	No of accessions	Accession codes
		RAPD	
I	1A	3	1 and 3
	1B	2	2
	1C	2	5 and 15
	1D	3	6 and 10
	1E	1	7 and 9
	1F	2	8
	1G	2	12 and 13
	1H	1	14 and 16
	1I	2	17
	1J	1	4 and 11
	1K	1	19
II	II	1	18
		ISSR	
I	1A	1	1
	1B	3	3,5 and 15
	1C	2	16 and 19
	1D	2	6 and 7
	1E	2	8 and 10
	1F	1	14
	1G	1	11
	1H	1	9
	1I	1	17
	1J	2	12 and 13
	1K	1	18
II	II	1	2 and 4
		RAPD+ISSR	
I	1A	2	1 and 3
	1B	1	2
	1C	2	5 and 15
	1D	1	16
	1E	1	11
	1F	2	12 and 13
	1G	1	17
	1H	1	6
	1I	3	7, 8 and 9
	1J	2	10 and 14
	1K	1	19
	1L	1	4
II	II	1	18

Table 4A.7 Origin wise distribution of turmeric variety in the RAPD, ISSR and RAPD-ISSR combined clusters

	Assam	Arunachal	Manipur	Meghalaya
RAPD	1A,1B,1C,1J	1C,1D,1E,1H	1C,1G,1H	1H, 1I,1K,II
ISSR	1A,1B,II	1B,1D,1E,1H	1B,1G,1J	1C,1I,1K
RAPD+ISSR	1A,1B,1C,1L	1C,1F,1H	1C,1F,1J	1D,1G,1K, II

4A.3.3.2 *Intra-varietal level*

The genetic similarity amongst the variety of each state was found to be varying using two marker system and their combinations tried. The similarity matrix for all variety has been shown (Table 4a.7, Table 4a.8 and Table 4a.9). For the turmeric variety of Assam, the highest and lowest genetic similarity was observed between the variety 2 and 3 (DICE genetic similarity 0.933) and 2 and 4 (0.462), respectively in the RAPD based clusters. On the contrary, the highest and the lowest genetic similarity was observed between the variety 4 and 5 (0.923), 3 and 4 (0.541), respectively. In the RAPD-ISSR combined analysis, the highest genetic similarity of 0.917 was observed between the variety 1 and 3, whereas the lowest genetic similarity of 0.519 was observed between the variety 3 and 4. The mean genetic similarity for the five varieties of Assam was found to be 0.69, 0.73 and 0.68 for RAPD, ISSR and RAPD-ISSR respectively.

Among the variety of Arunachal Pradesh, the maximum genetic similarity was observed to 0.634 (8 and 10) and the minimum genetic similarity was observed to be 0.435 (6 and 7) by RAPD markers. The maximum and the minimum values for ISSR marker system were experimentally found to be 0.979 (6 and 7), 0.864 (6 and 9) respectively. Similarly the maximum and minimum values of RAPD-ISSR combination was found to be 0.865 (7 and 8) and 0.744 (9 and 10), respectively. The variety of Arunachal Pradesh showed the average genetic similarity of 0.59, 0.93 and 0.81 by usage of RAPD, ISSR and RAPD-ISSR based markers respectively (Table 4a.7, Table 4a.8 and Table 4a.9).

The variety of Manipur showed the maximum genetic similarity of 0.778 (12, 13 and 12, 14) and the minimum genetic similarity of 0.50 (11 and 13) based on RAPD based study. The maximum similarity of 0.973 (12 and 13) and minimum similarity of 0.78 (12 and 14) was observed by the ISSR based study. The combination of RAPD and ISSR based study showed the maximum genetic similarity of 0.877 (12 and 13) and the minimum similarity of 0.701 (11 and 15). The mean genetic similarity for RAPD, ISSR and RAPD-ISSR marker system was found to be 0.65, 0.85 and 0.76, respectively (Table 4a.7, Table 4a.8 and Table 4a.9).

The highest and the lowest values of genetic similarity were observed to be within the variety of Meghalaya by RAPD based markers which was 0.647 (16 and

17) and 0.400 (16 and 18), respectively. With ISSR marker the values of genetic similarity was 0.878 (16 and 19) and 0.718. The maximum and the minimum estimate of genetic similarity were observed to be 0.722 (16 and 19) and 0.580 (16 and 18) respectively by the combination of RAPD and ISSR markers. The average genetic similarity values for RAPD, ISSR and RAPD - ISSR marker system were found to be 0.51, 0.77 and 0.66, respectively (Table 4A.7, 4A.8 and 4A.9).

4A.3.3. 3 *Genetic identity and genetic distances*

The Nei's unbiased measures of genetic identity and genetic distance among the varieties were also calculated (Table 4A.11). The genetic similarity values of turmeric varieties of Assam and Arunachal, Assam and Manipur, Assam and Meghalaya origin were found to be 0.86, 0.87 and 0.82 by RAPD analysis. The genetic similarity values were found to 0.89 and 0.83 between the varieties of Arunachal Pradesh and Manipur, Arunachal Pradesh and Meghalaya. The genetic similarity between Manipur and Meghalaya populations were found to be 0.87.

4A.3.4 Cophenetic correlation coefficient

The separation approach as revealed by the Mantel test comparing the results of RAPD, ISSR, and RAPD-ISSR indicated a significant correlation among the 19 turmeric varieties. The cophenetic correlation coefficients between cophenetic correlation matrix and the similarity matrix of the same marker system were also significant for RAPD ($r = 0.82$), ISSR ($r = 0.86$), and RAPD-ISSR ($r = 0.87$), supporting a good degree of confidence in the association obtained for the turmeric varieties of NE India. Mantel test yielded results ranging from good fit to very good fit for cophenetic values ($0.82 < r < 0.87$).

Table 4A.8 Distance matrix values based on RAPD data for *Curcuma longa* varieties

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	1.0																		
2	0.650	1.0																	
3	0.933	0.667	1.0																
4	0.578	0.462	0.500	1.0															
5	0.619	0.667	0.634	0.585	1.0														
6	0.512	0.486	0.476	0.476	0.462	1.0													
7	0.640	0.500	0.571	0.531	0.435	0.681	1.0												
8	0.600	0.455	0.571	0.531	0.478	0.596	0.778	1.0											
9	0.565	0.400	0.578	0.444	0.429	0.605	0.800	0.800	1.0										
10	0.537	0.629	0.500	0.400	0.432	0.684	0.667	0.662	0.537	1.0									
11	0.432	0.581	0.389	0.556	0.485	0.412	0.537	0.488	0.541	0.438	1.0								
12	0.537	0.571	0.550	0.450	0.541	0.421	0.533	0.533	0.537	0.556	0.625	1.0							
13	0.537	0.571	0.550	0.400	0.486	0.421	0.578	0.667	0.634	0.611	0.500	0.778	1.0						
14	0.488	0.514	0.500	0.450	0.541	0.579	0.667	0.711	0.683	0.722	0.563	0.778	0.722	1.0					
15	0.500	0.647	0.513	0.564	0.722	0.432	0.500	0.591	0.450	0.514	0.516	0.686	0.629	0.686	1.0				
16	0.500	0.529	0.513	0.462	0.500	0.486	0.591	0.636	0.650	0.514	0.581	0.686	0.686	0.800	0.706	1.0			
17	0.450	0.471	0.462	0.308	0.389	0.486	0.545	0.500	0.600	0.457	0.516	0.571	0.629	0.571	0.529	0.647	1.0		
18	0.333	0.333	0.343	0.400	0.375	0.424	0.300	0.350	0.389	0.258	0.370	0.323	0.258	0.387	0.383	0.333	0.400	1.0	
19	0.486	0.516	0.500	0.500	0.364	0.412	0.439	0.439	0.486	0.375	0.500	0.500	0.563	0.438	0.452	0.516	0.516	0.444	1.0

Table 4A.9 Distance matrix values based on ISSR data for *Curcuma longa* varieties

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	1.0																		
2	0.800	1.0																	
3	0.902	0.744	1.0																
4	0.588	0.769	0.541	1.0															
5	0.816	0.634	0.923	0.571	1.0														
6	0.809	0.667	0.840	0.606	0.792	1.0													
7	0.833	0.700	0.863	0.588	0.776	0.979	1.0												
8	0.800	0.667	0.868	0.556	0.784	0.939	0.960	1.0											
9	0.844	0.703	0.833	0.645	0.739	0.864	0.889	0.894	1.0										
10	0.833	0.700	0.902	0.588	0.816	0.936	0.958	0.960	0.933	1.0									
11	0.826	0.684	0.816	0.625	0.809	0.889	0.913	0.875	0.837	0.913	1.0								
12	0.810	0.765	0.756	0.714	0.744	0.732	0.762	0.727	0.821	0.762	0.850	1.0							
13	0.791	0.743	0.783	0.690	0.773	0.762	0.791	0.756	0.800	0.791	0.878	0.973	1.0						
14	0.809	0.718	0.840	0.606	0.750	0.913	0.936	0.939	0.864	0.936	0.889	0.780	0.810	1.0					
15	0.792	0.650	0.902	0.588	0.939	0.809	0.792	0.800	0.756	0.833	0.826	0.810	0.837	0.809	1.0				
16	0.783	0.632	0.816	0.563	0.851	0.756	0.783	0.792	0.791	0.826	0.818	0.800	0.780	0.800	0.870	1.0			
17	0.698	0.686	0.783	0.690	0.818	0.857	0.837	0.800	0.750	0.837	0.829	0.757	0.789	0.810	0.837	0.780	1.0		
18	0.683	0.667	0.727	0.593	0.714	0.700	0.683	0.651	0.684	0.683	0.615	0.686	0.667	0.650	0.683	0.718	0.778	1.0	
19	0.791	0.629	0.783	0.621	0.818	0.714	0.698	0.711	0.700	0.744	0.732	0.703	0.684	0.762	0.837	0.878	0.737	0.722	1.0

Table 4A.10 Distance matrix values based on RAPD-ISSR data for *Curcuma longa* varieties

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	1.0																		
2	0.725	1.0																	
3	0.917	0.707	1.0																
4	0.582	0.585	0.519	1.0															
5	0.725	0.649	0.796	0.579	1.0														
6	0.667	0.579	0.674	0.533	0.644	1.0													
7	0.735	0.595	0.720	0.554	0.611	0.830	1.0												
8	0.700	0.558	0.725	0.541	0.639	0.771	0.865	1.0											
9	0.703	0.545	0.710	0.526	0.591	0.736	0.842	0.845	1.0										
10	0.697	0.667	0.725	0.486	0.651	0.824	0.817	0.800	0.744	1.0									
11	0.651	0.638	0.635	0.588	0.675	0.684	0.736	0.697	0.700	0.718	1.0								
12	0.675	0.667	0.659	0.559	0.650	0.582	0.644	0.629	0.675	0.667	0.750	1.0							
13	0.667	0.657	0.674	0.522	0.642	0.600	0.682	0.711	0.716	0.709	0.712	0.877	1.0						
14	0.659	0.622	0.688	0.521	0.659	0.762	0.804	0.830	0.776	0.843	0.753	0.779	0.769	1.0					
15	0.659	0.649	0.733	0.575	0.847	0.643	0.652	0.702	0.612	0.699	0.701	0.753	0.744	0.756	1.0				
16	0.651	0.583	0.682	0.507	0.699	0.634	0.689	0.717	0.723	0.691	0.720	0.747	0.737	0.800	0.800	1.0			
17	0.578	0.580	0.635	0.471	0.625	0.684	0.690	0.652	0.675	0.667	0.694	0.667	0.712	0.701	0.701	0.720	1.0		
18	0.519	0.508	0.557	0.484	0.568	0.575	0.494	0.506	0.541	0.500	0.515	0.515	0.478	0.535	0.535	0.580	0.667	1.0	
19	0.650	0.576	0.659	0.554	0.623	0.579	0.571	0.581	0.597	0.587	0.638	0.609	0.629	0.622	0.676	0.722	0.638	0.603	1.0

Table 4A.11 Inter-population genetic similarity and genetic distances evaluated by 9 RAPD and 7 ISSR primers for turmeric accessions

	Population 1 (RAPD/ISSR/ RAPD-ISSR)	Population 2 (RAPD/ISSR/ RAPD-ISSR)	Population 3 (RAPD/ISSR/ RAPD-ISSR)	Population 4 (RAPD/ISSR/ RAPD-ISSR)
Population 1	-	0.86/0.79/ 0.83	0.87/ 0.91/ 0.89	0.82/ 0.86/ 0.84
Population 2	0.15/0.24/ 0.18	-	0.89/ 0.85/ 0.87	0.83/ 0.77/ 0.81
Population 3	0.14/0.24/ 0.12	0.12/0.16/ 0.13	-	0.87/ 0.89/ 0.89
Population 4	0.19/0.15/ 0.17	0.19/0.26/ 0.21	0.14/0.11/ 0.12	-

4A.3.5 Principal component analysis (PCA)

PCA is a frequently used technique to detect varietal structure and understand evolutionary history (Crawford 1990). PCA is a method that summarizes high dimensional genetic data into plots with minimum loss of information. The plots created are believed to show the genetic relationship among the varieties. PCA was originally applied to human population genetic studies as an alternative method to phylogenetic trees. The relationship between human populations cannot be analyzed with phylogenetic trees properly, because several populations can be derived from a single population and gene flow is very common. These problems can be avoided with PCA and other related methods, such as correspondence analysis and multidimensional scaling analysis. The first three components of principal component analysis accounts for 32 %, 12 % and 9 % (RAPD), 44 %, 15 %, and 9 % (ISSR), 40 %, 11 %, 8.0 % (RAPD-ISSR) markers respectively. So the cumulative contribution of the first three components varied from 53 % (RAPD), 68 % (ISSR), and 59 % (RAPD-ISSR) respectively.

Although the information contribution of the ISSR-PCA was higher, RAPD based PCA could able to discriminate the turmeric varieties. The RAPD-PCA showed four different clusters of the varieties according to the state of origin, whereas the groupings of the ISSR based PCA demarcated the 19 varieties into two different groups in the second dimension of PCA. The first group consisted of the varieties of Assam and Meghalaya; the second group consisted of the varieties of Manipur and Arunachal Pradesh. The RAPD-ISSR based PCA analysis grouped all varieties into three clusters (Arunachal and Manipur, Manipur and Meghalaya, Assam and Mizoram) (Fig 4a.3).

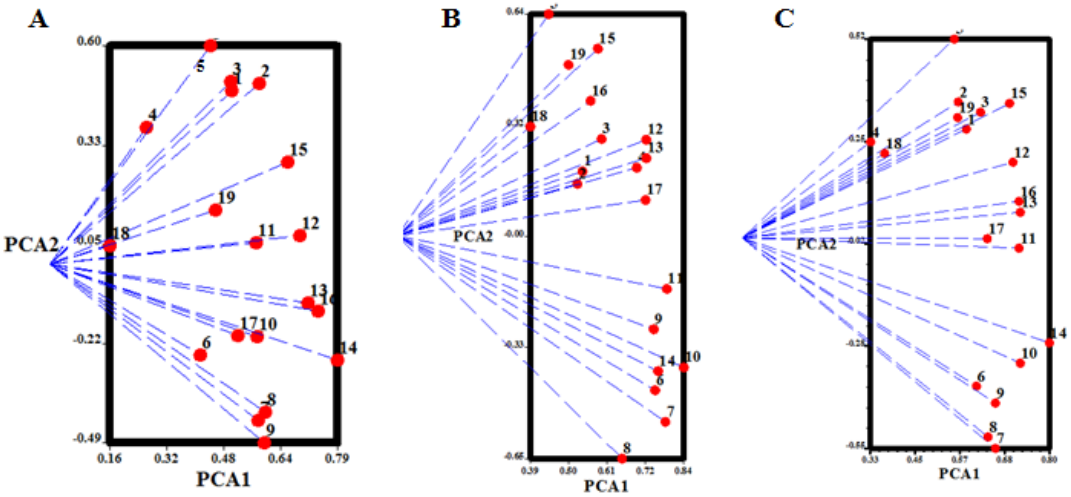

Fig 4A.3 PCA plot of turmeric cultivated varieties based on **A.** RARD, **B.** ISSR and **C.** RAPD -ISSR marker.

4A.4 Conclusion

Precise information of genetic variability among turmeric varieties of the NE India is important for establishing core collections of germplasm and in aiding breeding work. On account of the low productivity in NE states compared to the rest of the states of India, proper initiative should be taken on the suitable spread of the existing germplasms among the farmers so as to encompass the turmeric productivity. Among the 19 studied varieties, the use of RAPD and ISSR markers has been demonstrated to be a powerful tool to define how turmeric genetic diversity is structured in NE India, leading to a better management of the germplasm bank and also to promote the use of these varieties in future breeding programs. The results presented here are the first step towards better knowledge of the germplasm available in NE India and can help to guide future studies of the crop.

CHAPTER 4B

MOLECULAR PHYLOGENY OF *HEDYCHIUM* AS DISSECTED USING PCA ANALYSIS AND HIERARCHICAL CLUSTERING

This chapter discusses the genetic relationship patterns of *Hedychium*

MOLECULAR PHYLOGENY OF *HEDYCHIUM* AS DISSECTED USING PCA ANALYSIS AND HIERARCHICAL CLUSTERING

4B.1 Introduction

Hedychium J. König (Zingiberaceae) is an economically important genus, consisting of 65 species worldwide of which Northeast (NE) India has the highest species concentration (24 out of 65) (Wood et al 2000; Hamidou et al 2008). *Hedychium* species are widely cultivated for their perfume, and as a useful raw material for manufacturing paper. Moreover, some species are cultivated for their edible flowers (He 2000) and because of horticultural significance. The inflorescence of *Hedychium coronarium*, the national flower of Cuba, is aromatic and showy, resembling a cluster of flying white butterflies. Flowering in *Hedychium* mainly occurs in summer and autumn, while a few species bloom in winter and spring. The medicinal and ornamental importance of the genus has been well documented. The medicinal efficacies of the essential oil extracted from leaves, flowers and rhizomes of these plants including cercaricidal properties (Warren & Peters 1968), molluscicidal activity (Saleh et al 1982), potent inhibitory action with IC (50) against leukotriene biosynthesis (Kumar et al 1982), antimicrobial activities (Medeiros et al 2003; Gopanraj et al 2005), *in-vitro* pediculicidal activity (Jadhav et al 2007), anti-inflammatory and analgesic effects (Shrotriya et al 2007) are well established.

In spite of the ornamental, medicinal significance, limited knowledge is available for many members of *Hedychium*. In addition, high intra and interpopulation variation has led to debate concerning species concepts and boundaries. Also, the genus is facing the loss of genetic diversity, due to uncontrolled uprooting of the rhizome and the whole plant in huge quantities for preparation of traditional medicine. Species level taxonomy of Zingiberaceae propagating vegetatively is unresolved because morphological differences are inconclusive due to the short lived flowers and

the plants flower during monsoon season. So there is an urgent need for sustainable management to conserve the germplasm; however sustainable management has so far not succeeded further aggravating the extinction. For any future analysis using *Hedychium*, it may be practical to have information regarding the genetic relatedness of all wild species. The data on genetic similarity is a mandatory requirement to study the evolutionary history of a species, as well as for other studies, such as intra-specific variations, genetic resources conservation, etc.

Phylogenetic relationship plays an important role in parental selection. In the traditional taxonomy of *Hedychium*, the taxonomic characters are bract arrangement, number of flowers per bract and petal colours. The species appears to have some correlation with the intensity of fragrance and its sugar content of nectar. Though the highest sugar content is measured in *H. elatum* which is moderately fragrant, the range was high in the species with lesser fragrance such as *H. coccineum, H. rubrum, H. stenopetalum, H. thrysiforme and H. villosum* while the lower range of sugar content was measured in *H. coronarium, H. chrysoleucum* and *H. urophyllum* which are intensely fragrant. This might be indicative of the way; each species attracts its pollinators. Further studies need to be conducted in this regard to understand its pollination biology (Sarangthem et al 2012). The Eastern Himalayan biodiversity 'hotspot' which has been currently scaled up as Indo Burma hotspot is, the second largest and next only to Mediterranean basin. So it is quite obvious that the differentiation will be more in *Hedychium* of NE India. *Hedychium* reproduces sexually through dispersal of seeds by birds and asexually by spreading rhizomes. For *H. coronarium, H. gardnerianum and H. flavescens*, sexual reproduction has been reported (Csurhes & Jones 2008). But for other species, field observations indicate that although the plants flower almost every year, seed production is limited, and seedling establishment is poor. Propagation, therefore, appears to occur mostly by vegetative means. The facultative xemogamy is seen in some of the *Hedychium* members (Wang et al 2004) whereas the self-sterility commonly occurs within the genus (Gao et al 2005). Such characters simply offered each species a relative, but arbitrary location in the genus, without any quantification of similarity. The quantification of similarity, however, can be easily realized by using molecular techniques. Molecular markers are used as the taxonomic aid because of advantages

of high polymorphism, non-pleotropy and clear identifying alleles, etc. Molecular data sets can provide useful information for addressing various aspects of plant taxonomy. The major challenge associated with any molecular method is to determine the appropriate taxonomic level at which it is most informative and to correlate it with morphologically definable taxonomic groupings. Random amplified polymorphic DNA (RAPD) markers were introduced in 1990 (Williams et al 1990). Their use as molecular markers for taxonomic and systematic analyses of plants (Bartish et al 2000), as well as in their breeding and genetic relationships has increased exponentially (Ranade et al 2001) due to easiness of the procedure. Apparently, RAPD markers evolve more quickly than the isozyme based method. RAPD uses small amounts of the sample for analysis and detects variation in both coding and non-coding regions of the genome while morphological and isozyme variations only reflect difference in protein-coding sequences (Adams 1999; Sharma & Jana 2002). Similarly, inter simple sequence repeat (ISSR) markers are useful in detecting genetic polymorphisms between and within the species by generating a large number of markers that target multiple micro satellite loci distributed across the genome (Dasuki et al 2000). Among many researchers, amplified fragment length polymorphism (AFLP) is the marker technology of choice since it combines the reliability of classical restriction-based fingerprinting with the speed and convenience of polymerase chain reaction (PCR)-based marker techniques (Vos et al 1995; Powell et al 1996; Ude et al 2000 a, b; Lu et al 2002). The AFLP technique rapidly generates hundreds of highly replicable DNA markers, thus allowing high resolution genotyping (Loh et al 2000). These DNA fingerprinting techniques have been used successfully for confirmation of relationship of *Boesenbergia* (Vanijajivaa et al 2005); phylogenetic analysis in *Amomum* (Kaewsri et al 2007); genetic relationship in *Curcuma* (Das et al 2011) etc. There is only one report on the phylogenetic analysis and genetic mapping of Chinese *Hedychium* using sequence related amplified polymorphism (SRAP: Gao et al 2008). Therefore, the present study was undertaken to investigate the intra- as well as inter-species genetic relationship using principal component analysis and hierarchical clustering among the species of the *Hedychium* occurring in NE India by PCR based molecular markers.

4B.2 Materials and methods

4B.2.1 Plant material

The materials for the present study consisted of 11 species of the genus *Hedychium*, wildly collected from different locations of NE India (Fig 4B.1; Table 4B.1). Each species collected in duplicates were maintained in the departmental green house of IIT Guwahati, Assam. Geographical positions of the collection locations are shown in Fig 4B.1. Herbarium vouchers are deposited in the herbarium GUBH (recognized by American Botanical Society), with duplicates in IIT Guwahati, Assam. References for identification and distribution were also made with various flora's and articles (Chaturvedi & Moaakum 2008; Wu & Larsen 2000; Picheansoonthon & Wongsuwan 2008; Rao & Verma 1969, 1972). The classified species include *H. coronarium* J. Koenig, *H. chrysoleucum* Hook, *H. coccineum* Buch.-Ham., *H. dekianum* A.S.Rao & Verma, *H. flavescens* Carey ex Roscoe, *H. stenopetalum* Lodd, *H. marginatum* Clarke.

4B.2.2 DNA extraction and subsequent RAPD-PCR and ISSR-PCR amplification

For details section 4A.2.2 and section 4A.2.3 can be referred.

4B.2.3 AFLP analysis

AFLP analysis was performed with minor modifications (Vos et al 1995). Five hundred nanograms of DNA was double digested with *Eco*R1 and *Mse* I, and then ligated with adapters using T4 DNA ligase (New England Biolabs). The pre-selective-PCR product was diluted in a ratio of 1:10 with TE buffer and then used as a template for the selective amplification. The pre-selective amplification was performed at 72 ^{o}C for 2 min followed by 20 cycles of denaturation (at 94 ^{o}C for 20 s), primer annealing (at 56 ^{o}C for 30 s) and primer extension (at 72 ^{o}C for 2 min) and finally maintained at 60 ^{o}C for 30 min. An aliquot of the pre-selective PCR product was electrophoresed on 1.5% agarose gel and checked for amplification.

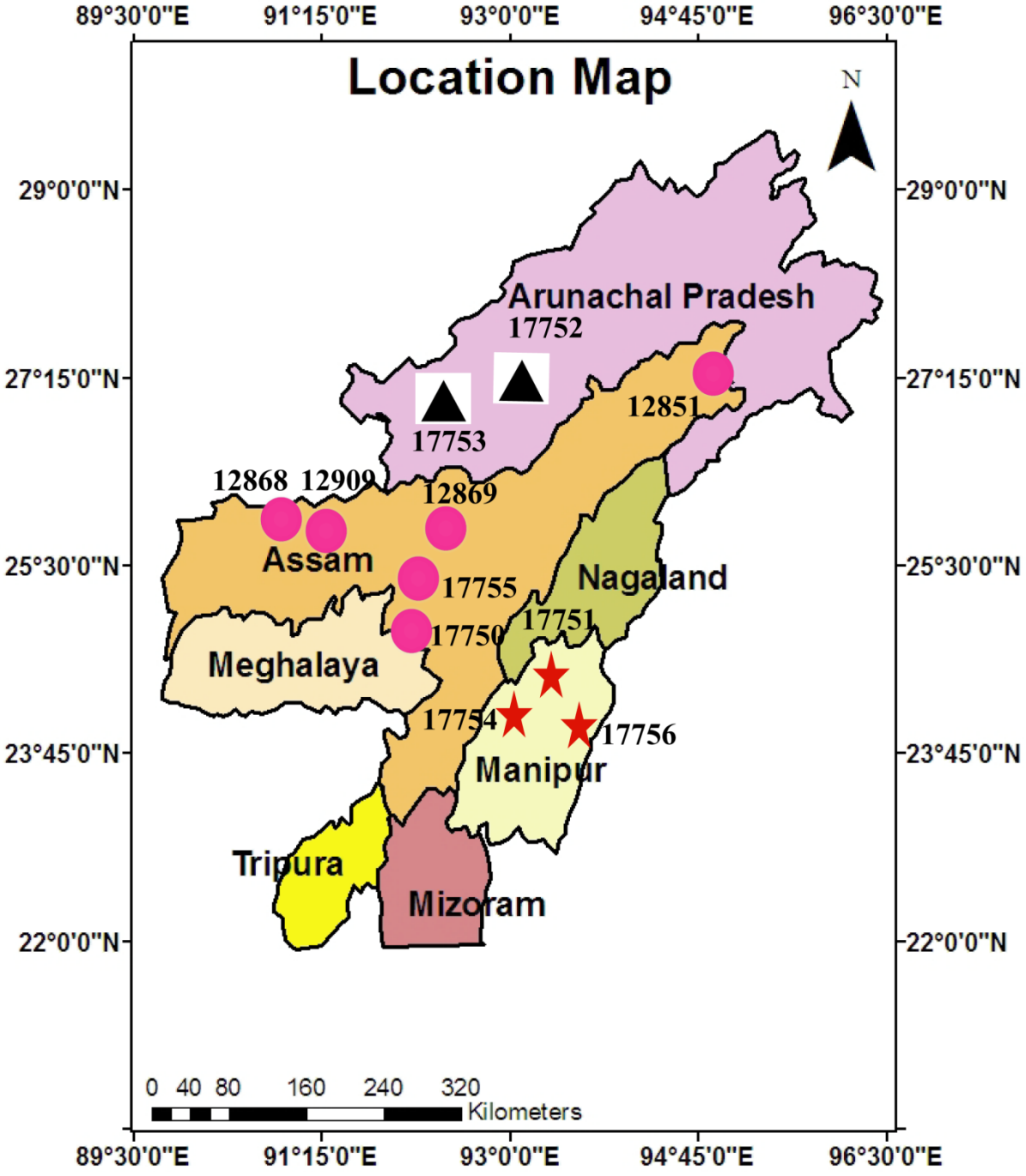

Fig. 4B.1. Geographical distribution of the *Hedychium* species studied. The samples have been shown in the figures by the voucher numbers. *H. coronarium* J. Koenig (17755); *H. dekianum* A.S.Rao & Verma (12868); *H. flavum* Roxb. (12909); *H. stenopetalam* Lodd (12869); *H. spicatum* Lodd (17750); *H. chrysoleucum* Hook (12851); *H. gardnerianum* Wall. ex Spreng (17753); *H. marginatum* Clarke (17754); *H. flavescens* Lodd (17752); *H. aurantiacum* Wall (17751); 11. *H. coccineum* Wall (17756).

Table 4B.1 List of *Hedychium* species used in the study with their morphological information and habitat description

S. No.	Species Name	Voucher no	Morphological description	Habitat
1	*Hedychium coronarium* J. Koenig	17755	*Leaves:* oblong or oblong-lancealote; *Bracts:* large oblong imbricate; *Flowers:* white	Swamps and wet meadows.
2	*H. dekianum* A.S.Rao & Verma	12868	*Leaves:* oblong or oblong-lancealote ; *Bracts:* large oblong *Flowers:* white	Lowlands shaded areas.
3	*H. flavum* Roxb.	12909	*Leaves:* oblong or oblong-lancealote; *Bracts:* large oblong *Flowers:* yellow	Humid and shaded areas.
4	*H. stenopetalam* Lodd.	12869	*Leaves:* oblong or oblong-lancealote; *Bracts:* oblong *Flowers:* light orange	Humid and shaded areas.
5	*H. spicatum* Lodd.	17750	*Leaves:* oblong or oblong-lancealote; *Bracts:* large oblong *Flowers:* white	From moderately to highly wet areas.
6	*H. chrysoleucum* Hook.	12851	*Leaves:* oblong or oblong-lancealote; *Bracts:* large oblong; *Flowers:* white tinged with yellow.	Humid and shaded areas.
7	*H. gardnerianum* Wall. ex Spreng.	17753	*Leaves:* oblong, white pulverulent beneath; *Bracts:* large oblong *Flowers:* bright lemon yellow	Windward hilly slopes.
8	*H. marginatum* Clarke	17754	*Leaves:* oblong or oblong-lancealote; *Bracts:* oblong *Flowers:* Yellow flower with reddish-brown inflorescence	Windward moist hilly slope.
9	*H. flavescens* Lodd.	17752	*Leaves:* oblong or oblong-lancealote; *Bracts:* large oblong imbricate *Flowers:* sulphur yellow	Windward hilly slope.
10	*H. aurantiacum* Wall.	17751	*Leaves:* long oblong; *Bracts:* oblong ; *Flowers:* bright orange yellow	Foot hills receiving heavy rainfall.
11	*H. coccineum* Wall.	17756	*Leaves:* lanceolate ; *Bracts:* oblong; *Flowers:* small bright crimson	Foot hills and moist grassland.

It was then diluted (1:20) with DNase free water and used as a template for the selective amplification. The selective amplification was performed using primers from the AFLP selective primer kit. The selective amplification involved the following thermal cycling conditions: denaturation at 94 ^{o}C for 2 min followed by 11 cycles of 94 ^{o}C for 20 s, 66 ^{o}C for 30 s, and 72 ^{o}C for 2 min. The annealing temperature was reduced by 1 ^{o}C every cycle until it reached 56 ^{o}C. This was followed by another 20 cycles of amplification at 94 ^{o}C for 20 s, 56 ^{o}C for 30 s and 72 ^{o}C for 2 min, with a final extension at 60 ^{o}C for 30 min. The PCR product of selective amplification (1 µL) was mixed with 0.5 µL of the GeneScan 500 ROX internal size standard (Applied Biosystems P/N 402985) and 8.5 µL of Hi-Di Formamide (Applied Biosystems P/N 4311320). The mixture was then denatured prior to separation by capillary gel electrophoresis on an automated DNA sequencer (ABI 3130, Applied Biosystems). The electropherograms generated by the sequencer were interpreted with Gene scan software. Fragments sized from 50 to 500 base pairs (bp) with a peak height >50 bp in the electropherogram were retained for subsequent analysis.

4B.2.4 Data analysis

The detailed data analysis of the RAPD, ISSR and AFLP markers has been mentioned in section 4a.2.4. Hierarchical clustering (HCA) was further created for *Hedychium*, where each profile is a string with 11 entries (number of species analyzed). HCA was performed with the Cluster (v. 3.0) and visualized with Tree View (Eisen et al 1998). Species in the study were clustered according to the similarity of their phylogenetic profiles and if there was a similarity of one species to that of the other species, the value 1 was assigned at that position (red), if not, 0 was assigned (black).

4B.3 Results and discussion

4B.3.1 Efficiency of polymorphism detection

A total of 6 species in two replicates was used for the intra-species diversity study of *Hedychium*. Using 5 selected RAPD primers and ISSR primers, a study was conducted to know whether there exist any variation in the intra-species level or not. The average intra-species polymorphism was found to be 40 % within the individuals of *Hedychium*. The highest intra-species genetic polymorphism (% P = 33.33; Ne = 1.24; *I* = 0.20) was found within the replicates of *H. coronarium*. The lowest intra-species genetic polymorphism (% P = 1.85; Ne = 1.01; I= 0.01) was found within the replicates of *H. chrysoleucum*. The intra-

species genetic polymorphism (% P = 5.56; N_e= 1.01; *I* = 0.01) was of the similar magnitude within the species of *H. stenopetalam, H. spicatum, H. gardnerianum* and *H. flavescens.* The intra-species population differentiation (G_{ST}) was observed to be 0.85. An indirect estimate of the number of migrants per generation (N_m = 0.08) indicated that low gene flow exist among the individuals (Table 4b.2). The number of loci and their genome coverage are critical in obtaining reliable estimates of intra-species genetic relationship among replicates of *Hedychium*. The correlation between the original similarity matrix and cophenetic values for the RAPD-ISSR studies was found to be 0.96. This signifies that higher congruence was obtained among the phenetic classification based on the combined marker data. The intra-species variation in terms of percentage of polymorphism, observed number of alleles, effective number of alleles, Nei's gene diversity and Shannon's information index was found to be the highest in *H. coronarium* and to be the lowest in *H. chrysoleucum*. This might be the result of cross pollination in *H. coronarium* and vegetative propagation in other individuals of the species. The average intra-species polymorphism was found to be 40 %. *Hedychium* species undergo hybridization resulting in high level of intra-species population differentiation (G_{ST} = 0.80). Since the habitats of the *Hedychium* are discrete and their chemical characters are not well discriminated, low genetic divergence might be expected.

The inter-species relationship was studied in triplicates for the 11 *Hedychium* species of NE India. Of 20 RAPD primers, 11 gave successful amplification with a total of 158 bands, of which 131 were polymorphic (82.9 %). The number of polymorphic bands varied from 5 (OPD 07) to 23 (OPD 03) with an average of 11.9 per primer. The size of the amplified fragments varied from 250 to 2000 bp. The polymorphic information content (PIC) varied from 0.31 (OPD 19) to 0.47 (OPD 11) with an average PIC of 0.38. Marker Index (MI) varied from 23.13 (OPD 07) to 47.00 (OPD 11) with an average MI of 31.71. The most informative primer was OPD 11. Of the 20 ISSR primers, 15 gave successful amplification with a total of 141 bands, of which 131 were polymorphic with the polymorphism of 92.9 %. The highest number of polymorphic fragments obtained was for HB 12 (12) and the lowest for 17898B (4) with an average of 9.00 per primer. The polymorphic information content (PIC) was varied from 0.29 (811) to 0.45 (HB13) with an average PIC of 0.37. Five AFLP primer combinations produced a total of 416 polymorphic fragments with 100 % polymorphism. PIC was varied from 0.27 (*Mse*I-CAC/*Eco*RI-ACT) to 0.32 (*Mse*I-CAG/*Eco*RI-ACT) with an average value of 0.29. The MI varied from 27 (*Mse*I-CAC/*Eco*RI-ACT primer combination) to 32 (*Mse*I-CAG/*Eco*RI-ACT primer combination) (Table 4B.3).

Table 4B.2 Genetic diversity parameters estimated by RAPD and ISSR primers for intra-species study in *Hedychium*

Species	Sample size (n)	Observed no of alleles (Na)	Effective no of alleles (Ne)	Nei's gene diversity (h)	Shannon's Information Index (I)	% P	G_{ST}	Nm (G_{ST})
RAPD + ISSR								
H. coronarium	2	1.33 ± 0.48	1.24 ± 0.34	0.14 ± 0.01	0.20 ± 0.02	33.33		
H. chrysoleucum	2	1.01 ± 0.14	1.01 ± 0.09	0.01 ± 0.00	0.01± 0.00	1.85		
H. stenopetalam	2	1.05 ± 0.23	1.03 ± 0.16	0.02 ±0.01	0.03 ±0.00	5.56		
H. spicatum	2	1.05 ±0.23	1.03 ± 0.16	0.02 ±0.01	0.03 ±0.01	5.56		
H. gardnerianum	2	1.05 ±0.23	1.03 ± 0.16	0.02 ±0.01	0.03 ±0.01	5.56		
H. flavescens	2	1.05 ±0.23	1.03 ± 0.16	0.02 ±0.01	0.03 ±0.01	5.56		
Intra-species	12	1.92 ± 0.26	1.60 ± 0.30	0.19 ± 0.14	0.09 ± 0.19	40.00	0.85	0.08

P= percentage of polymorphic loci; G_{ST} = diversity among individuals; Nm = gene flow $0.25 (1 - G_{ST}) / G_{ST}$

Table 4B.3 Degree of polymorphism and polymorphic information content for inter-species genetic relationship in *Hedychium*

RAPD Primer						ISSR Primer					
Primer Code No	TNB	NPB	% P	PIC	MI	Primer Code No	TNB	NPB	% P	PIC	MI
OPA01	16	12	75.0	0.39	29.25	17898B	5	4	80.0	0.39	31.20
OPA02	11	9	81.0	0.36	29.16	826	11	11	100.0	0.35	35.00
OPD02	26	21	80.0	0.32	25.60	HB12	13	13	100.0	0.39	39.00
OPD03	29	23	79.0	0.39	30.81	HB13	8	7	87.5	0.45	39.37
OPD05	8	7	87.5	0.40	35.00	17899A	11	11	100.0	0.34	34.00
OPD07	8	5	62.5	0.37	23.13	816	9	9	100.0	0.38	38.00
OPD10	7	7	100.0	0.37	37.00	817	9	8	88.8	0.40	35.52
OPD11	6	6	100.0	0.47	47.00	811	10	6	60.0	0.29	17.40
OPD18	15	13	86.6	0.40	34.64	825	12	12	100.0	0.33	33.00
OPD19	16	16	100.0	0.31	31.00	807	9	9	100.0	0.45	45.00
OPD20	16	12	75.0	0.35	26.25	824	10	9	90.0	0.33	29.70
Total	158	131	926.6	4.13	348.84	HB15	7	6	85.7	0.33	28.28
Mean	14.36	11.90	82.90	0.38	31.71	17899A	6	5	83.3	0.42	34.98
AFLP Primer						814	12	12	100.0	0.39	39.00
MseI-CAC/EcoRI-ACT	111	111	100	0.27	27	809	9	8	88.8	0.39	34.63
MseI-CAG/EcoRI-ACT	46	46	100	0.32	32	**Total**	141	131	1364.10	5.24	514.08
MseI-CAT/EcoRI-ACT	110	110	100	0.30	30	**Mean**	9.71	9.00	92.9	0.37	34.27
MseI-CTC/EcoRI-ACT	51	51	100	0.29	29						
MseI-CTG/EcoRI-ACT	98	98	100	0.28	28						
Total	416	416	500	1.46	146						
Mean	83.2	83.2	100	0.292	29.2						

TNB: Total number of bands, NPB: Number of polymorphic bands, % P: Percentage of polymorphic bands, PIC: Polymorphic information content, MI: Marker Index.

Inter-species variation study in *Hedychium* showed increased level of discrimination of ISSR markers, may be due to comparatively high marker index over AFLP markers. The PIC was found to be higher in RAPD markers compared to ISSR and AFLP markers. The genome coverage is more in ISSR as compared to RAPD and AFLP markers. These findings coincide with the observation of previous study from our lab on genetic relationship of *Curcuma* species from North East India using dominant molecular markers (Das et al. 2011).

4B.3.2 Correlation of gene diversity and mean genetic similarity for genetic relationship

The gene diversity was estimated for 11 *Hedychium* species using the data sets of the three marker types separately. The genetic diversity was found to be uniform for RAPD and ISSR and AFLP makers (Shannon's information index I = 0.48), although combination of RAPD and ISSR markers resulted in increased genetic diversity (I = 0.50) (Table 4B.4). The DICE similarity coefficient (genetic similarity) increased for AFLP markers (0.68) compared to ISSR (0.55), RAPD (0.53), and RAPD-ISSR (0.54) markers respectively. The combination of RAPD-ISSR marker resulted in genetic similarity which is the average of that of RAPD and ISSR markers separately. But more combination (RAPD-ISSR-AFLP) resulted in decreased mean Dice similarity co-efficient (Fig. 4B.2). The relationship between genetic diversity (Shannon's information index, I) and mean genetic similarity (Dice's coefficient) was measured by Pearson product-moment correlation coefficient. The result showed a positive correlation (r = 0.46) between mean DICE genetic similarity and genetic diversity.

Table 4B.4 Comparison of genetic diversity in *Hedychium* species by different markers

Parameter	Markers			
	RAPD	ISSR	RAPD + ISSR	AFLP
The number of observed alleles, na	2.0000 ± 0.0000	1.9934 ± 0.0811	2.0000 ± 0.0000	2.0000 ± 0.0811
The mean number of effective alleles, ne	1.5183 ± 0.3026	1.5317 ± 0.3209	1.5569 ± 0.3145	1.5183 ± 0.3209
The mean Nei's gene diversity index, h	0.3141± 0.1396	0.3163 ± 0.1495	0.3298 ± 0.1404	0.3141 ± 0.1495
Shannon index, I	0.4805± 0.1712	0.4809 ± 0.1847	0.4993± 0.1690	0.4805± 0.1847

4B.3.3 Genetic similarity analysis

The first genetic relationship for *Hedychium* species of NE India has been presented, producing trees and PCA plot with high topological support for most nodes using RAPD-

ISSR and AFLP markers. The UPGMA, hierarchical clustering and PCA analysis of combined RAPD-ISSR and AFLP datasets represents our best hypothesis of genetic relationships among *Hedychium*. We recommend the use of RAPD-ISSR and AFLP topology as the most complete framework for future studies on phylogenetic comparative analyses, tests of bio-geographic hypotheses and models for trait evolution. Given the correlation of similarity matrix data from different markers, we therefore prefer to combine them in analyses, because this approach generally helps to overcome errors or introgression at one locus, and to increase explanatory power (Huelsenbeck et al 1996; Edwards et al 2000; Nixon & Carpenter 2005). Although we consider the concatenated RAPD-ISSR dataset to provide the best current representation of the evolutionary history of *Hedychium*, further analyses should attempt to verify our findings through additional sampling of inter-species lineages.

Cluster analysis and PCA are valuable for determining relationship among individuals of the same and different species (Crawford 1990). The current study was conducted to investigate the intra-species and inter-species variation of *Hedychium*. The combined data set of RAPD-ISSR UPGMA clustering and PCA analysis showed low genetic variation to reveal the genetic structure of *Hedychium* species, which provided some insight into the species status. This is because in the RAPD-ISSR combined UPGMA dendrogram, the replicates were grouped together in the same cluster. The highest and the lowest Nei's genetic identity between the species was observed to be 0.83 (*H. stenopetalum* and *H. spicatum*) and 0.44 (*H. stenopetalum* and *H. gardnerianum*), respectively. Cluster I of the dendrogram consisted of species of *H. coronarium, H. chrysoleucum, H. flavescens, H. stenopetalam* and *H. spicatum*. Cluster II of the dendrogram consisted of solitary species *H. gardnerianum* (Fig 4b.3).

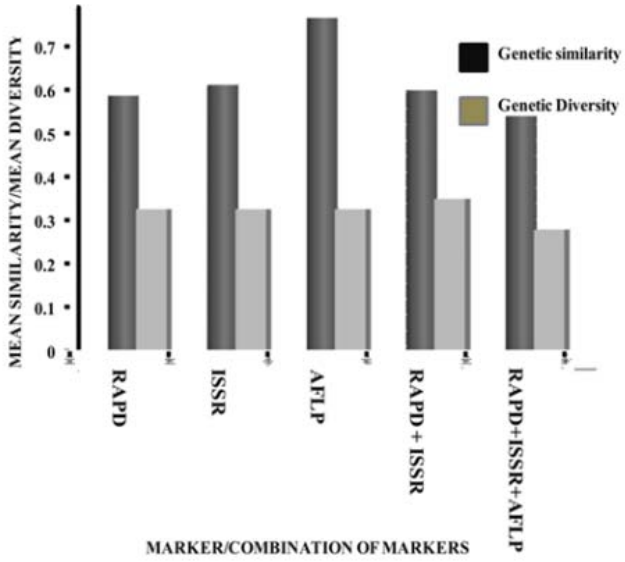

Fig. 4B.2 Mean genetic similarity and genetic diversity observed for 11 *Hedychium* species acquired from different locations of NE India. It is based on the DICE similarity coefficient and Shannon Information Index as (*I*) estimated by the RAPD, ISSR and AFLP markers independently as well as in combinations.

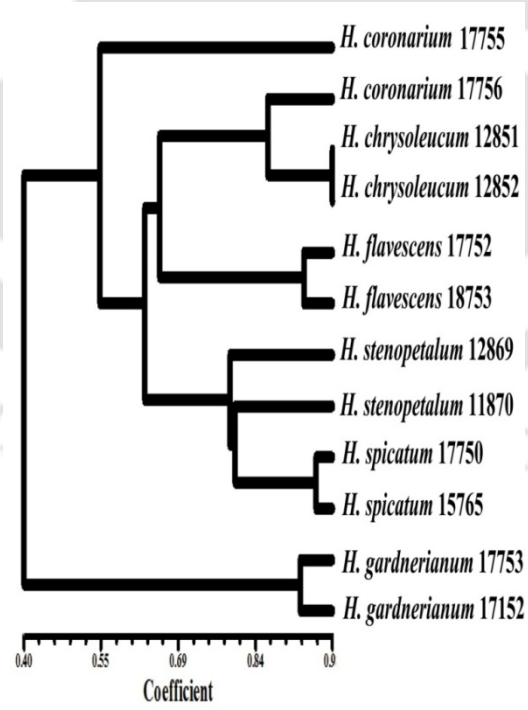

Fig 4B.3 The UPGMA dendrogram (RAPD-ISSR combined markers) for intra species genetic relationship of *Hedychium* species based on the combined RAPD-ISSR data set.

The DICE genetic similarity values derived from the RAPD-ISSR data ranged from 0.48 (*H. flavescens* and *H. dekianum*) to 0.79 (*H. coccineum* and *H. aurantiacum*). Similarly AFLP showed the lowest genetic similarity coefficient between *H. chrysoleucum* and *H. stenopetalam* (0.521) while the highest value was calculated between *H. coccineum* and *H. aurantiacum* (0.79). This result showed that the inter-species genetic similarity of the genus *Hedychium* are almost same using the PCR based molecular markers. The dendrogram constructed on RAPD-ISSR combined binary matrix grouped in two clusters at the level of similarity of 0.55. Cluster I consisted of 8 species and the cluster II of 3 species. Cluster I of RAPD-ISSR combined dendrogram formed 2 subgroups. Subgroup I consisted of *H. flavum, H. dekianum, H. coronarium, H. chrysoleucum* and sub group II consisted of *H. stenopetalam, H. spicatum, H. coccineum* and *H. aurantiacum*. The sub group I further segregated in two sub groups. The group I consisted of *H. flavum* and *H. dekianum*. The group II consisted of *H. coronarium* and *H. chrysoleucum*. The similarity of *H. flavum* and *H. dekianum* is 0.693. *H. coronarium* is related to *H. chrysoleucum* at the level of similarity of 0.670. The sub group II of cluster I of RAPD and ISSR combined dendrogram consisted of four species viz; *H. stenopetalam, H. spicatum, H. coccineum* and *H. aurantiacum*. The maximum similarity (0.787) was observed for *H. coccineum* and *H. aurantiacum*. The Cluster II consisted of three species segregated into two sub groups. Sub group I of cluster II consisted of two species (*H. marginatum* and *H. gardnerianum)* with the similarity coefficient of 0.73. The sub group II of cluster II consisted of the solitary species (*H. flavescens)* with the similarity coefficient of 0.551 and 0.614 with *H. marginatum* and *H. gardnerianum*, respectively.

Dendrogram constructed based on AFLP similarity matrix, showed two distinct groups for the 11 species of *Hedychium* at the level of similarity of 0.60, placing *H. flavescens* and *H. marginatum* in cluster II and the rest of the species in the cluster I. *H. flavescens* and *H. marginatum* had 63 % similarity between them. Cluster I formed two subgroups. Sub group I consisted of *H. flavum, H. coronarium, H. dekianum* and *H. chrysoleucum.* Sub group II of cluster I consisted of *H. spicatum, H. stenopetalam, H. coccineum, H. aurantiacum* and *H. gardnerianum.* The level of similarity between *H. coccineum* and *H. aurantiacum* was found to be 0.790, which is of similar magnitude to that of the RAPD-ISSR (0.787) combined dendrogram. The species clustering of RAPD-ISSR combined dendrogram was similar to AFLP dendrogram in the sub group I of cluster I (Fig 4B.4). The amount of red area was more in RAPD-ISSR and AFLP hierarchical clustering

compared to the other dendrograms. This signifies the tree formed by hierarchical clustering of RAPD-ISSR and AFLP dendrograms are the most informative compared to RAPD, ISSR and RAPD-ISSR-AFLP dendrograms (Fig 4B.4).

Cluster I comprised of species *H. flavum, H. dekianum, H. coronarium, H. chrysoleucum, H. stenopetalam* and *H. spicatum* which were collected from moist, shaded lowlands. *Hedychium* species bearing, strong aroma, fleshy rhizome, large oblong bract were grouped in cluster I. Morphological similarity of small epiphytes with robust fleshy rhizome between *H. coronarium* and *H. dekianum* justifies their placement in the same groups. *H. coccineum* and *H. aurantiacum* possess morphological similarity of oblong bract size and the morphological distinction of flower colour (bright crimson: *H. coccineum* and bright orange yellow: *H. aurantiacum*). *H. gardnerianum* and *H. flavescens* shared a similarity of 70 %. *H. flavescens* have the large oblong imbricate bract whereas *H. gardnerianum* has a large oblong bract. The flower colour of *H. flavescens* was sulphur yellow and the flower colour of *H. gardnerianum* was bright lemon yellow. The other species of Cluster II had oblong bract with yellow flower with a reddish brown inflorescence (*H. marginatum*). In the UPGMA dendrogram of RAPD-ISSR and AFLP markers, species of similar altitude have been grouped together. Our study of PCR based molecular markers is slight deviation from the marker analysis by other researchers who split *Hedychium* into three groups in terms of the species distribution according to altitude; each group correlated to a separate climatic zone (Wood et al 2000). Based on the morphological and molecular markers information, the 11 species are clearly differentiated. *H. chrysoleucum* is considered by some author as synonym of *H. coronarium* while others consider it as a hybrid called *H. coronarium* 'Gold Spot' due to the yellow patch at the base of labellum. However, in this study both are treated as separate species. *H. chrysoleucum* is known to the people of Manipur as ''Takhellei'' while *H. coronarium* is known as ''Loklei'' and the rhizomes of both the species are consumed as vegetable. *H. coronarium, H. chrysoleucum, H. flavum, H. dekianum, H. stenopetalum, H. spicatum, H. coccineum* and *H. aurantiacum* are restricted to the plains. Based on this data, the genus appears to have a distinct differentiation in the distribution of its species between the hills and plain regions. *H. Marginatum* and *H. flavescens* are found to grow mainly on hilly slopes with its foliage hanging downwards, while its inflorescence curved upwards. Chinese *Hedychium* was grouped into two clusters based on SRAP markers (Gao et al 2008). The first group consisted of *H. densiflorum, H. convexum, H. spicatum* and *H. yunnamense.* The second cluster consisted of three sub-groups of 18 species. The highest similarity

percentage (97 %) was reported between *H. flavescens* and *H. chrysoleucum.* The discrepancy of our study from the earlier reported study by SPAR based marker was the positioning of *H. spicatum* which was found to the cluster with *H. stenopetalam.* Our study also revealed that *H. flavescens* was close to that of *H. gardnerianum* with the similarity percentage of 59 %. It was also evident that RAPD-ISSR clustering approach closely follows the grouping of *H. coronarium, H. flavum, H. coccineum* into the same sub group of cluster II.

4B.3.4 PCA

In the present study inter-species genetic relationship among 11 species of *Hedychium* was analyzed by PCA, cluster analysis of 11 RAPD, 15 ISSR and 5 AFLP primer combinations displaying differential grouping patterns. The highest cumulative contribution of the first three principal components to total variation was found to be by RAPD-ISSR markers compared to their individual use. The highest percent contribution of Eigen values of RAPD-ISSR combined marker system is implicating better information of relatedness for the species studied. PCA analysis of RAPD-ISSR and AFLP data-set for inter-species genetic relationship showed two discrete clusters (Fig 4b.5) which is in good agreement with the cluster analysis of RAPD-ISSR and AFLP markers.

The Eigen values were found to be higher in the two dimensional plot when the combination of marker system was attempted. The representation of the collections from different altitudes and intensity of flower aroma was separated into two distinct clusters in the combined RAPD-ISSR dendrogram and the AFLP dendrogram. In the RAPD-ISSR combined dendrogram, species of the moist lowlands with strongly fragrant flower (*H. coronarium, H. dekianum, H. flavum, H. chrysoleucum, H. stenopetalam, H. spicatum, H. marginatum, H. coccineum, H. aurantiacum*) were combined in one cluster. A small cluster consisting of species of *H. flavescens* and *H. gardnerianum* possessed geographic similarity of high altitude and have yellow and mildly fragrant flowers. The AFLP-PCA analysis closely follows the altitude based clustering of RAPD-ISSR derived PCA.

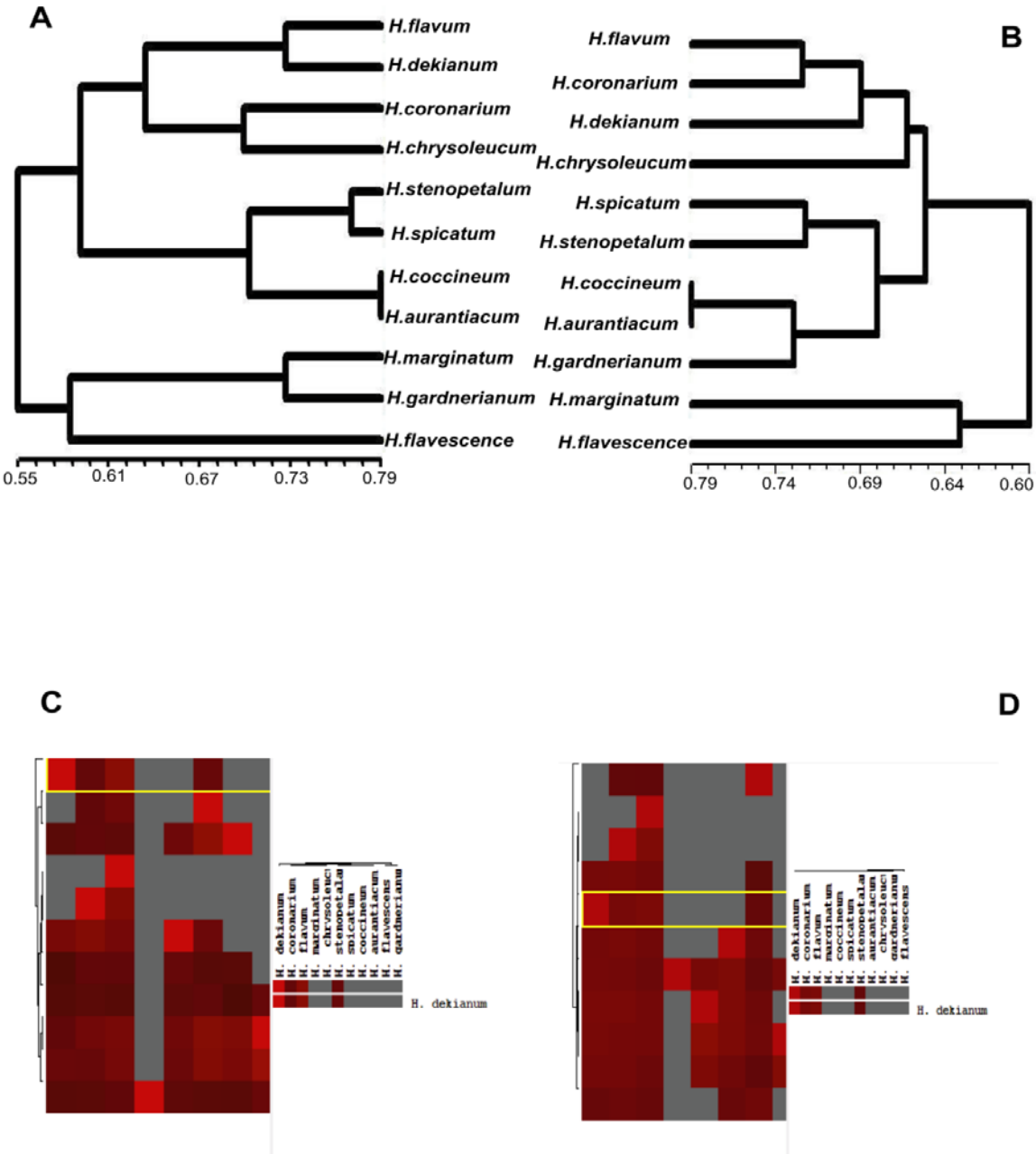

Fig 4B.4 Phylogenetics and Hierarchical Clustering. UPGMA dendogram of the A**.** RAPD-ISSR; **B.** AFLP maker system; **C.** HCA of all of the samples by RAPD-ISSR; **D.** HCA of all of the samples by AFLP**.**

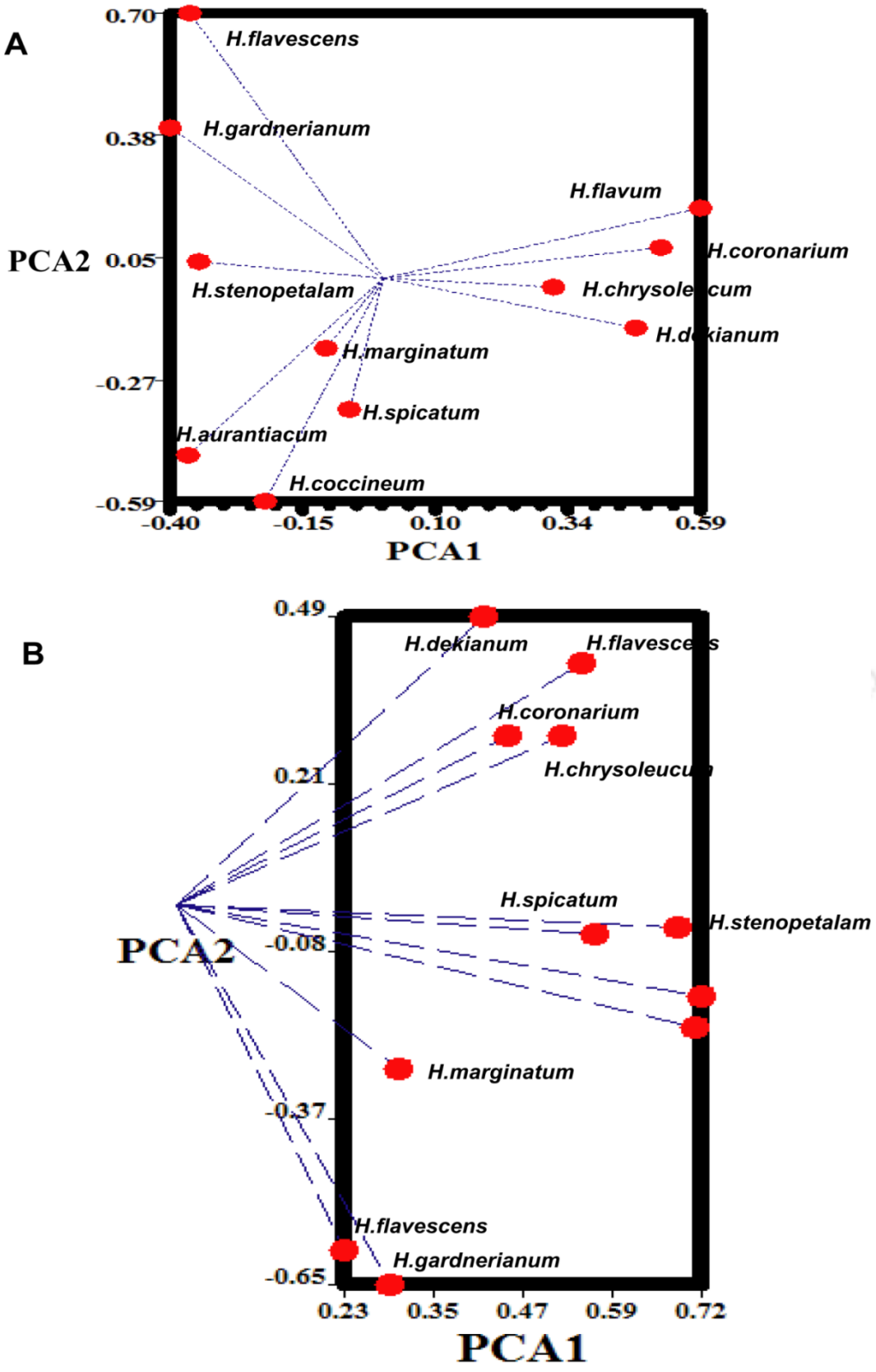

Fig 4B.5 PCA plots of *Hedychium* species of NE India based on molecular markers**. A.** RAPD-ISSR **B.** AFLP. PCA analysis of RAPD-ISSR combined data-set and AFLP segregate species collected from different altitude and flower aroma into two groups represented by dotted lines.

4B.3.5 Correlation coefficient for inter-species study

The separation approach as revealed by the Mantel test comparing the results of RAPD, ISSR and AFLP indicated a significant correlation among the different *Hedychium* species. The cophenetic correlation coefficients between cophenetic correlation matrix and the similarity matrix of the same marker system were also significant for RAPD (r = 0.89), ISSR (r = 0.86), and AFLP (r = 0.83), supporting a good degree of confidence in the association obtained for the species of *Hedychium*. Mantel test yielded results ranging from good fit to very good fit of cophenetic values (0.83< r < 0.89). Still the correlation values was higher in RAPD (r = 0.89) compared to ISSR (r = 0.86) and AFLP (r = 0.83) markers. The correlation among the similarity and cophenetic matrices of different markers are shown in Table 4B.5. Correlation coefficient of the similarity matrix was higher in the RAPD-ISSR dendrogram (r = 0.48) followed by ISSR-AFLP (r= 0.06), and the RAPD-AFLP (r= -0.26) dendrogram, respectively. The highest correlation coefficient of RAPD-ISSR combined marker system (r = 0.93) gave us the confidence of finding the phylogenetic relationships among the species of *Hedychium* (Fig 4b.6). This was one of the reasons we combined the binary matrix of RAPD and ISSR markers to decipher genetic relationship of 11 *Hedychium* species. Although cophenetic correlation coefficient between the similarity and cophenetic matrices of AFLP maker was found to be 0.83, the clustering pattern closely followed the RAPD-ISSR clustering. Nevertheless the additive similarity matrix (RAPD-ISSR-AFLP) resulted in inconclusive patterns in the UPGMA dendrogram, PCA analysis and the hierarchical clustering.

Mantel test indicated that the combination of PCR based markers were better compared to the independently used marker system used to study the genetic relationships among 11 *Hedychium* species. This result was supported by the low correlation coefficient among three markers with various degrees of fit. These results suggest that the manner of polymorphism differs because of marker specificity. Modest correlation between marker systems was also reported in wheat (Bohn et al 1999), soybean (Powell et al 1996), maize (Pejic et al 1998), and safflower (Sehgal & Raina 2005). In addition, the relation is assumed to depend on the genome coverage and sequence type recognized by each marker system (Powell et al 1996; Pejic et al 1998; Sehgal & Raina 2005). Different dendrograms and PCA plot were obtained by the independent marker systems with no conclusive common groupings detected. The PCA, UPGMA and HCA of combined marker systems are complementing each other, as they are based on different mathematical models and approaches to data

analysis.The combined usage of the marker systems resulted in grouping of the species based on altitude. The little discrepancy obtained by RAPD-ISSR and AFLP markers justifies that each marker represents only a part of the genome causing the congruence of the dendrogram.

Table 4B.5 Comparison of correlation coefficient between similarity matrices and co-phenetic matrices derived from different markers

Cophentic correlation matrix	Similarity matrix values		
	RAPD	ISSR	AFLP
RAPD	0.89	0.46	-0.26
ISSR	0.48	0.86	0.06
AFLP	-0.02	-0.02	0.83

Note. Above diagonal values represent correlation coefficients between similarity matrices and below diagonal values represent correlation coefficients between co-phenetic matrices. Values on the diagonal represent co-phenetic correlation for markers

4B.4 Conclusion

The PCA and cluster analyses of a binary matrix (RAPD-ISSR and AFLP) showed a unique genetic structure in *Hedychium*. The possible hindrances to the geographical distribution of *Hedychium* are the mountains (Himalaya, Patkai Hills), rivers (Bramhaputra and Barak River in Assam) and invasive human development. These obstacles directly prevent the species from dispersal. Cluster and PCA analysis of 11 species of *Hedychium* samples generated two relatively well-defined clusters that contained most of the samples. The first group consisted of *H. coronarium* and *H. spicatum* derived by hybridization between the *Hedychium* seed parent and a pollen plant from another species. The strong aroma of the flowers may be the evolutionary derived trait among the species. The small cluster (*H. flavescens* and *H. gardnerianum*) showed low genetic differentiation among the species thus exaggerating the taxonomic differentiation of lightly fragrant flowers from the cluster I. Therefore, RAPD, ISSR and AFLP are reliable methods for calculating genetic relationships reflecting coding and non-coding regions of the genome and they could well be used in aiding identification as well as classification of the Zingiberaceae using more species in each genus.

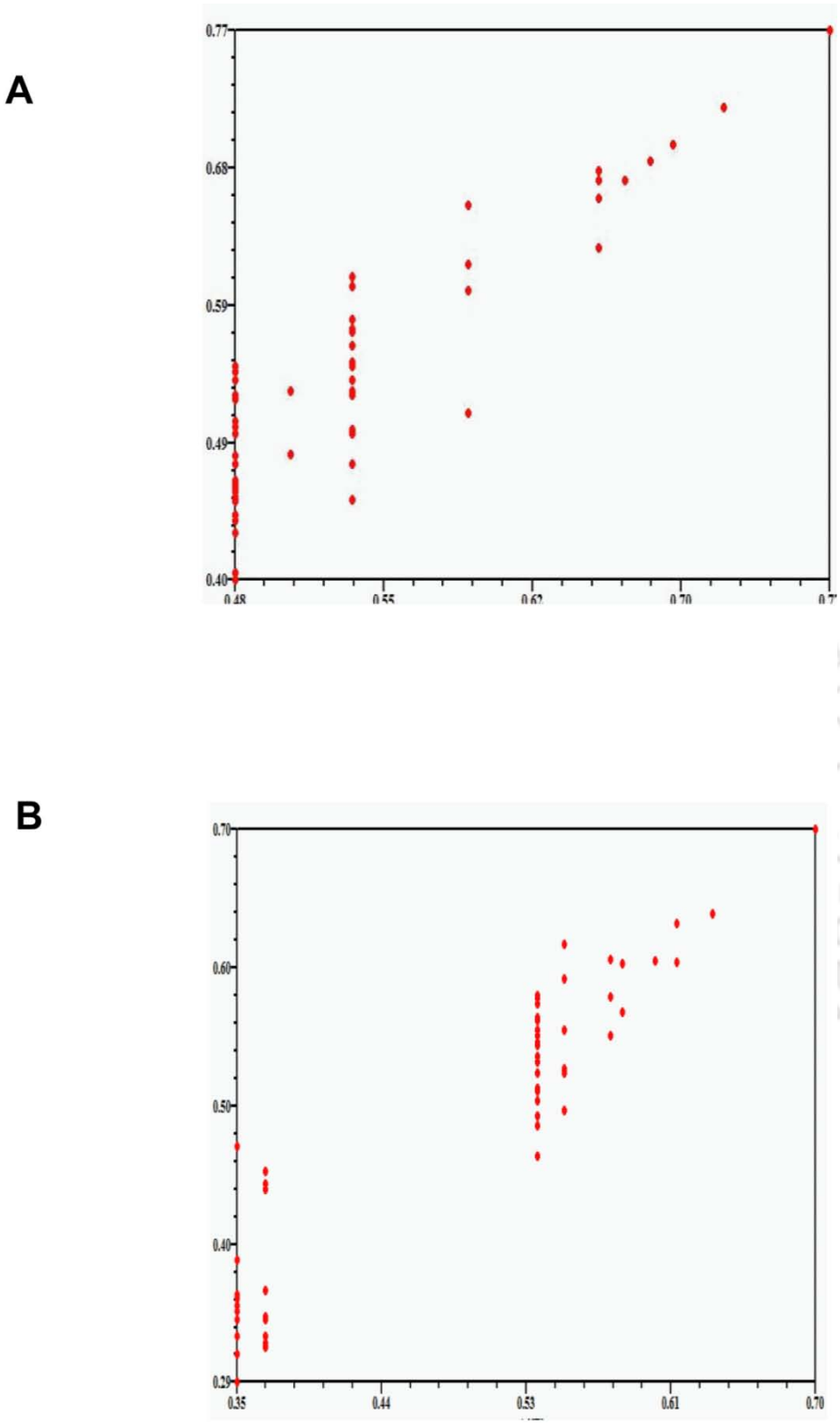

Fig. 4B.6 Matrix correlation of two way Mantel test of similarity and cophenetic matrices. **A.** RAPD-ISSR (r = 0.93) and **B.** AFLP (0.83).

Further study on single nucleotide polymorphisms (SNPs) together with morphological data, chromosome study and nuclear DNA content of more species should reduce any confusion in the species identification and help to provide a better understanding of evolutionary phylogeny, especially when combined with a study on gene specific analysis. Phylogenetic and phylogeographic study will be improved with the increase in the number of nuclear, mitochondrial and plastid loci and the incongruent signals among loci could be minimized. The incongruent signals may be due to the horizontal gene transfer, gene duplication, hybridization or introgression and lineage sorting. This study confirmed that multiple marker utilization in combinations (RAPD-ISSR) can be crucial for estimation of the relatedness of *Hedychium* at the inter-species level.

CHAPTER 4C

GENETIC RELATIONSHIP OF *KAEMPFERIA* BASED ON MOLECULAR MARKERS

This chapter discusses the genetic relationship patterns of *Kaempferia*

GENETIC RELATIONSHIP OF *KAEMPFERIA* BASED ON MOLECULAR MARKERS

4C.1 Introduction

Kaempferia L. comprises about 60 species geographically distributed from India to Southeast Asia (Sirirugsa, 1992; Larsen & Larsen, 2006), with a concentration of 22 species from British India (Baker 1890). It is a diverse polyploidy complex containing many taxa of economic, medicinal, ornamental and cultural importance. Baker (1890) listed four subgenera with a total of 22 species in his account on the genus *Kaempferia* for British India. Schumann (1903) considered the trilocular ovary with axile placentation a key character of the genus *Kaempferia*. Taxonomic classification of *Kaempferia* is tricky on account of the morphological similarity of vegetative parts amongst the species of the same genus and other genera in Zingiberaceae, such as *Boesenbergia*, *Cornukaempferia*, *Curcuma*, and *Scaphochlamys*. Taxonomic identification is withheld on account of the absence of the floral parts. The flowering season of *Kaempferia* is short, and inflorescences of some species (e.g., *K. rotunda*) appear before leafy shoots and last only for 1-2 weeks. In Northeast India, most *Kaempferia* species are dormant during November to early May. Therefore, a combination of several diagnostic characteristics, both vegetative and floral morphology, is essential for taxonomic decision at any species level.

Molecular phylogenetics have revitalized taxonomy and systematic of most living groups (Pyron & Wiens, 2011). DNA fingerprinting for evaluating the genetic relatedness among the species of the genera has become an essential tool for characterization and conservation of bio resources. The ease with which arbitrarily amplified dominant (AAD) markers such as RAPD, AFLP and ISSR could be used to generate vast quantities of data

led to their rapid application addressing a diverse range of biological questions. The most significant uses of AAD markers remain in genetic mapping, diagnostic fingerprinting and in the study of genetic structure within and between individual species. AAD markers may also be utilized for phylogenetics and systematics. Random amplified polymorphic DNA (RAPD; Williams et al 1990), inter simple sequence repeats (ISSR; Zietkiewicz et al 1994) and amplified fragment length polymorphism (AFLP; Vos et al 1995) have been extensively applied as an alternative genetic markers to allozymes and RFLPs for fingerprinting (Mueller & Wolfenbarger 1999), linkage mapping (Jones et al 1997), paternity analysis (Krauss 1999), gene flow (Waycott & Barnes 2001), elucidation of clones or hybrids (Waycott 1998; Hodkinson et al 2002), and population genetic analysis (Nybom & Bartish 2000; Nybom 2004). The phylogenetic evaluation of *Kaempferia* has been carried out using chloroplast DNA (*psbA-trnH* and *petA-psbJ*) sequences (Techaprasan et al 2010). The genetic relationships among *Boesenbergia* (Zingiberaceae), *Kaempferia* and *Scaphochlamys* was established by RAPD markers (Vanijajiva et al 2005). We used RAPD, ISSR and AFLP molecular markers to investigate the genetic relatedness of four species of *Kaempferia*.

4C.2 Materials and methods

4C.2.1 Plant material

The materials for the present study consisted of four species of *Kaempferia*, collected wildly from different locations of Assam (Table 4c.1). The species were maintained in the Departmental green house of IIT Guwahati, Assam.

Table 4C.1 List of *Kaempferia* species used in the study

S. No	Species name	Collected from	Voucher No	Latitude, Longitude and Altitude(in meter)	Leaf characters
1	*K. galanga* L.	Khetri (Assam)	12871	26° 11´ 49´´ N, 92°09´ 10´´E and 484	Slightly dark green shiny, wavy margin, acute apex - dorsal Rough ventral, light green wavy margin- ventral
2	*K. angustifolia* Roxb.	Jalukbari (Assam)	17767	26° 14´ 40´´N, 91° 67´ 12´´ E and 55	Dark green velvety texture, acute apex - dorsal. Rough light green- ventral
3	*K. pulchra* Ridl.	Jalukbari (Assam)	17766	26° 14´ 40´´N, 91° 67´ 12´´ E and 55	Dark green, velvety texture, patterned coloration (green) Slightly wavy margin, large in size, acute apex, green midrib – dorsal. Purple, parallel venations, hairy, velvety texture- ventral
4	*K. rotunda* Blanco.	NEDFi (Assam)	12870	26° 20´ 06´´N, 92° 93´ 75´´E and 58	Parallel venation, patterned coloration (green), regular margin, shiny – dorsal. Purple, parallel venation, hairy, velvety texture- ventral

4C.2.2 DNA extraction and subsequent RAPD and ISSR amplification

For details section 4A.2.2 and section 4A.2.3 can be referred.

4C.2.3 AFLP

For details section 4B.2.4 can be referred.

4C.2.4 Data analysis

The detailed data analysis of the RAPD, ISSR and AFLP markers mentioned in section 4A.2.4.

4C.3 Results

4C.3.1 Efficiency of polymorphism detection

A total of 4 species was used for the inter-species diversity study of *Kaempferia*. Using 8 AFLP primer combinations 614 fragments were detected out of which 518 were polymorphic (84.4 %). The number of polymorphic fragments varied from 27 (*Eco*RI-FAM-ACT & *MSe*I-CAA) to 120 (*Eco*RI-ACT-FAM & *MSe*I-CTT) with an average of 64.8 per primer. PIC was found to vary from 0.28 (*Eco*RI-FAM-ACT & *MSe*I-CAA, *Eco*RI-FAM-ACT & *MSe*I-CTA) to 0.34 (*Eco*RI-FAM-ACT & *MSe*I-CTC) with an average PIC of 0.31. The MI was found to vary 19.38 (*Eco*RI-FAM-ACT & *MSe*I-CAA, *Eco*RI-FAM-ACT & *MSe*I-CTA) to 29.4 (*Eco*RI-ACT-FAM & *MSe* I-CAT) with an average MI of 26.2 (Table 4c.2). Using 9 selected RAPD primers, a total of 81 bands were generated of which 80 bands were polymorphic (98.76%). The number of polymorphic bands varied from 4 (OPA02) to 17 (OPA12) with an average of 8.8 per primer. The size of the amplified fragments varied from 250 to 2000 bp. The polymorphic information content (PIC) varied from 0.32 (OPA16 and OPA18) to 0.46 (OPA20) with an average PIC of 0.36. The most informative primers were OPA 20. Using 11 selected ISSR primers, a total of 113 bands were generated of which 112 bands were polymorphic (99.10%). The number of polymorphic bands varied from 6 (17899B, 807) to 15 (824, 17898B) with an average of 10.18 per primer. The size of the amplified

fragments varied from 250 to 2000 bp. The polymorphic information content (PIC) varied from 0.30 (807) to 0.42 (17899B) with an average PIC of 0.38. The most informative primers were 17899B (Table 4C.3).

Table 4C.2 Degree of polymorphism and polymorphic information content for *Kaempferia* for using markers

Serial No	Primer combinations	Total number of bands	Polymorphism		PIC	MI
			Number	Percentage		
1	*Eco*RI-FAM-ACT & *MSe*I-CAA	39	27	69.23	0.28	19.38
2	*Eco*RI-FAM-ACT & *MSe*I-CAC	91	79	86.81	0.30	26.04
3	*Eco*RI-FAM-ACT & *MSe*I-CAG	56	44	78.57	0.32	25.14
4	*Eco*RI-ACT-FAM & *MSe*I-CAT	110	98	89.09	0.33	29.4
5	*Eco*RI-FAM-ACT & *MSe*I-CTA	41	29	70.73	0.28	19.80
6	*Eco*RI-FAM-ACT & *MSe*I-CTC	50	38	76.00	0.34	25.84
7	*Eco*RI-FAM-ACT & *MSe*I-CTG	95	83	87.36	0.31	27.08
8	EcoRI-ACT-FAM & *MSe*I-CTT	132	120	90.90	0.32	29.09
Total		**614**	**518**	**-**	**-**	**-**
Average			**-**	**84.36**	**0.31**	**26.15**

Table 4C.3 Degree of polymorphism and PIC for *Kaempferia* using RAPD and ISSR markers.

Serial No	Primer	TNB	NPB	% P	PIC	MI	Serial No	Primer	TNB	NPB	% P	PIC	MI
1	OPA02	6	6	100	0.42	42	1	17899A	14	14	100	0.35	35
2	OPA11	8	8	100	0.38	38	2	17899B	6	6	100	0.42	42
3	OPA12	18	17	94.44	0.39	36.83	3	807	7	6	85.71	0.303	25.97
4	OPA14	12	12	100	0.26	26	4	814	8	8	100	0.39	39
5	OPA16	13	13	100	0.32	32	5	824	15	15	100	0.39	39
6	OPA18	8	8	100	0.32	32	6	826	8	8	100	0.41	41
7	OPA17	4	4	100	0.36	36	7	844	8	8	100	0.36	36
8	OPA19	8	8	100	0.34	34	8	825	10	10	100	0.39	39
9	OPA20	4	4	100	0.46	46	9	17898A	11	11	100	0.4	40
							10	17898B	15	15	100	0.38	38
							11	811	11	11	100	0.41	41
	Total	81	80					**Total**	113	112			
	Mean	9	8.88	99.38	0.36	35.87		**Mean**	10.27	10.18	98.70	0.38	37.81

TNB: Total number of bands, NPB: Number of polymorphic bands, % P: Percentage of polymorphic bands, PIC: Polymorphic information content, MI: Marker Index

4C.3.2 Correlation of gene diversity and mean genetic similarity for genetic relationship study of Kaempferia

The gene diversity was estimated for all the *Kaempferia* species using the data sets of three marker types separately. The genetic diversity was found to be the highest for ISSR (Shannon's information index $I = 0.45$) followed by RAPD ($I = 0.40$) and AFLP ($I = 0.38$) markers (Table 4C.4).

Table 4C.4 Comparison of genetic diversity in four *Kaempferia* species by different markers

Parameter	Values		
	RAPD	**ISSR**	**AFLP**
The number of observed alleles, na	1.98 ± 0.16	1.99 ± 0.09	1.97 ± 0.02
The mean number of effective alleles, ne	1.38 ± 0.27	1.45 ± 0.27	1.22 ± 0.58
The mean Nei's gene diversity index, h	0.25± 0.13	0.28 ± 0.12	0.24 ± 0.52
Shannon index, I	0.40± 0.16	0.45 ± 0.52	0.38± 0.25

4C.3.3 Genetic relationship analysis as revealed by molecular markers at the inter-species level

The inter-species relationship among the four taxa belonging to the genera *Kaempferia* was studied by 8 AFLP primer combinations, 9 RAPD and 11 ISSR markers. A genetic similarity matrix was constructed from the AFLP, RAPD and ISSR profiling data, based on Dice coefficient. The tree formed by RAPD-ISSR combined marker gave the actual tree according to the morphology.

The combined dataset of RAPD-ISSR marker showed the maximum similarity ranged from 0.51 (*K. pulchra* and *K. angustifolia*) and the minimum similarity was observed for 0.280 between *K. galanga* and *K. pulchra*. *K. galanga* represented 33.6 %, 28.0 % and 32.7 % similarity with *K. rotunda, K. pulchra* and *K. angustifolia* (Fig 4C.1). This is also represented by the morphology. The leaf structure of *K. galanga* is semi-adpressed /elliptic in nature. The leaf structure of *K. pulchra* is erect/ elliptic in nature. The leaf structure of *K. angustifolia* is erect/elliptic oblong in nature. Similarly the leaf structure of *K. rotunda* is erect/ lanceolate in nature. The DNA sequencing studies of *psbA-trnH* and *petA-psbJ-psbL* region also suggested that *K. galanga* (clade A) is phylogenetically separated from *K. angustifolia* (clade B), *K. pulchra* (clade C), and *K. rotunda* (clade E) (Techaprasan et al 2010).

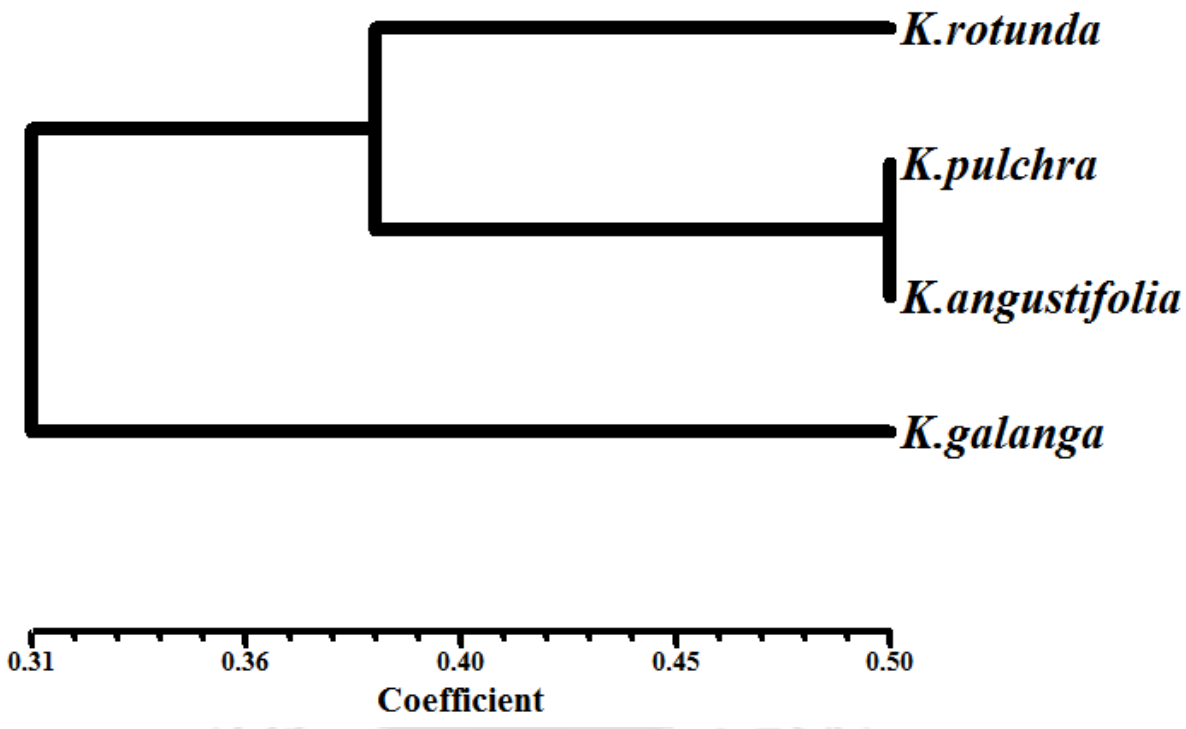

Fig 4C.1 UPGMA dendogram of RAPD - ISSR maker system.

4.4 Conclusion

The work revealed the unique phylogenetic structure in *Kaempferia*. Therefore, RAPD-ISSR marker combination reflected a reliable method for calculating genetic relationships since it reflects coding and non-coding regions of the genome and it could well be used in aiding identification as well as classification of the Zingiberaceae using more species in the genus. Further study on the single nucleotide polymorphism (SNP) together with gene content, morphological data and chromosome study of more species should reduce any confusion in the species identification and help to provide a better understanding of evolutionary phylogeny, especially when combined with a study on gene specific analysis.

CHAPTER 4D

INTER-GENERIC RELATIONSHIP OF ZINGIBEROIDEAE BY RAPD AND ISSR PRIMERS

This chapter discusses the inter-generic relationship of Zingiberoideae.

INTER-GENERIC RELATIONSHIP OF ZINGIBEROIDEAE BY RAPD AND ISSR PRIMERS

4D.1 Introduction

Genera like *Curcuma*, *Zingiber* and *Kaempferia* include edible species and have a productive export market worldwide. Zingiberaceae was also includes genera with ornamental importance (*Hedychium*) and with high medicinal value (*Alpinia*). Classifications of the family Zingiberaceae first proposed in 1889 and refined by others. Since that time four tribes have been recognized viz; (Globbeae, Hedychieae, Alpinieae and Zingibereae) based on morphological features, number of locules and placentation in the ovary, development of staminodia, modifications of the fertile anther, and rhizome-shoot-leaf orientation (Burtt & Smith 1972; Smith 1972). The family is sub divided into four sub families: Alpinoideae, Zingiberoideae, Siphonochiloideae and Tamijioideae. Alpinoideae has been characterised by ever green plants consisting of two tribes Alpinieae and Riedelieae. Zingiberoideae contains the previously recognised Hedychiae, Zingibereae and Globbeae (Kress et al 2002). The most recognizable floral features of this subfamily are the conspicuous and often well-developed lateral staminodes that are generally absent in the Alpinioideae. The most prominent vegetative feature that is universal in this subfamily is the parallel orientation of the plane of distichy of the leafy shoots with respect to the rhizome (perpendicular orientation in all other Zingiberaceae). Unlike the Alpinioideae and Tamijioideae, in which individuals are evergreen throughout the year, the Zingiberoideae, including taxa of the former Hedychieae, Globbeae and Zingibereae, have a forced dormancy period. Just prior to or at the earliest sign of the wet season, individuals will break dormancy with either vegetative shoots or reproductive shoots (e.g., *Hemiorchis*, *Gagnepainia*, *Mantisia*, some *Kaempferia* and many *Curcuma*). However, some species in the Zingiberoideae can also be evergreen in the wet forest habitats where they occur (e.g., some *Globbas*, *Zingibers*, and

Hedychiums), but these same species can be forced into dormancy under stress or in greenhouse environments (Kress et al 2002).

The study on intra-generic classification of Zingiberoideae by molecular markers is few (Kress et al 2002; Ngamriabsakul et al 2004; Vanijajiva et al 2005; Joshi et al 2012). In the present study, RAPD, ISSR profiling was used to assess the phylogenetic relationship among 18 prominent species of 5 genera of Zingiberaceae. This research represents the first genetic investigation of these species using RAPD and ISSR markers.

4D.2 Materials and methods

4D.2.1 Plant material

The plant material were collected from different parts of NE India during the field trip and maintained in replicates in the green house of IIT Guwahati. Table 4d.1 gives details of the plants used for the study.

4D.2.2 Out-group selection

Each phylogenetic study within the family Zingiberaceae prove that the tribe *Alpinieae* was the basal branch in the family followed by *Globbeae, Hedychieae* and *Zingibereae* (Soltis et al 2000; Wilf et al 2000; Wood et al 2000). Based on the previous studies, the out groups selected was *A. nigra* from the tribe *Alpinieae.*

Table 4D.1 Taxa used in this study with source and accession number

		Source	Accession no
	Out group		
1.	*Alpinia nigra*	Sivasagar	12911
	In group		
1.	*Zingiber cassumunar*	Rongia	12915
2.	*Kaempferia galanga*	Khetri	12871
3.	*K. angustifolia*	Jalukbari	17767
4.	*K. pulchra*	Jalukbari	17766
5.	*K. rotunda*	NEDFi	12870
6.	*Curcuma amada*	Amingaon	12951
7.	*C. angustifolia*	Khasi Hills	17745
8.	*C. aromatica*	Nagaon	17749
9.	*C. leucorrhiza*	Amingaon	17790
10.	*C. zedoaria*	Darrang	12859
11	*C.longa*	Barpeta	17765
12.	*Hedychium coronarium*	Guwahati	17755
13.	*H. flavum*	Arunachal Pradesh	12909
14.	*H. stenopetalam*	Guwahati	12869
15.	*H. spicatum*	Panbazar	12910
16.	*H. gardnerianum*	Bomdila	17753
17.	*H. ellipticum*	Silchar	17790

4D.2.3 In-group selection

For the in-group taxa, 17 species from 4 genera were used (Table 4D.1). One species of *Zingiber*, 4 species of *Kaempferia*, 6 species of *Curcuma* and 6 species of *Hedychium* were taken into consideration.

4D.2.4 DNA extraction and subsequent PCR amplification

The details of this section discussed in section 4A.2.2, 4A.2.3.

4D.2.5 Data analysis

The detailed data analysis of the RAPD and ISSR markers has been discussed in section 4A.2.4.

4D.3 Results

4D.3.1 *Efficiency of polymorphism detection*

A total of 4 RAPD and 4 ISSR primers gave successful amplification. A total of 38 and 26 polymorphic fragments (100 %) were obtained for 18 studied species for RAPD and ISSR markers, respectively. The highest (0.31) and the lowest (0.22) PIC values were obtained for OPA13 and OPA11 respectively with an average of 0.27. The highest (0.42) and the lowest (0.37) PIC values were observed in 817 and 811 respectively, with an average of 0.38 (Table 4D.2).

Table 4D.2 Degree of polymorphism and polymorphic information content for inter-generic study in Zingiberaceae

Primer code no	TNB	NPB	%P	PIC	MI
		RAPD			
OPA11	13	13	100	0.22	21.74
OPA13	9	9	100	0.31	31.07
OPA18	8	8	100	0.28	27.85
OPA20	8	8	100	0.27	27.46
Total/Mean	38	38	100	0.27	27.07
		ISSR			
17899B	8	8	100	0.38	37.73
811	5	5	100	0.33	33.46
809	6	6	100	0.38	37.65
817	7	7	100	0.42	42.15
Total/Mean	26	26	100	0.38	37.74

TNB: total no of bands; NPB: Number of polymorphic bands; % P: Percentage of polymorphic bands; PIC: Polymorphic information content; MI: Marker index

Although RAPD score over ISSR marker in generating total number of polymorphic bands, the average PIC and MI were higher in ISSR marker compared to RAPD markers. Genetic diversity estimated by ISSR markers was found to be higher compared to RAPD markers (Table 4D.3).

Table 4D.3 Genetic diversity parameters for inter-generic study in Zingiberaceae

Parameter	Markers		
	RAPD	ISSR	RAPD –ISSR
The number of observed alleles, na	2.00 ± 0.00	2.00 ± 0.08	2.00 ± 0.00
The mean number of effective alleles, ne	1.23 ± 0.21	1.44 ± 0.32	1.32 ± 0.26
The mean Nei's gene diversity index, h	0.17 ± 0.10	0.28 ± 0.15	0.22 ± 0.12
Shannon index, I	0.29± 0.14	0.44 ± 0.18	0.36 ± 0.17

4D.3.2 *Genetic similarity analysis*

A genetic similarity matrix was constructed from the RAPD, ISSR and RAPD-ISSR profiling data, based on DICE similarity coefficient for the 18 species. *C. leucorrhiza* and *C. zedoaria* showed the highest genetic similarity (0.933), and *K. angustifolia* and *H. coronarium* showed the least (0.100) for RAPD based marker analysis. The mean genetic similarity among all 18 genotypes was 0.33, 0.38 and 0.36 for RAPD, ISSR and RAPD-ISSR, respectively. The correlation coefficient of the similarity matrix derived from RAPD (0.88), ISSR (0.85) and RAPD+ISSR (0.85) data was calculated to detail the correlation of both types of molecular markers. The RAPD markers exhibited a better correlation value of 0.88, ($P \leq 0.001$) suggesting the suitability of RAPD markers for determining the genetic diversity of genotypes in the family Zingiberaceae. Similar study on the genetic diversity profiling of Zingiberaceae family by nucleotide binding site (NBS) profiling showed the choice of the markers based on correlation coefficients (Joshi et al 2012). So the phylogenetic interpretation of the Zingiberaceae family has been carried out by RAPD markers only.

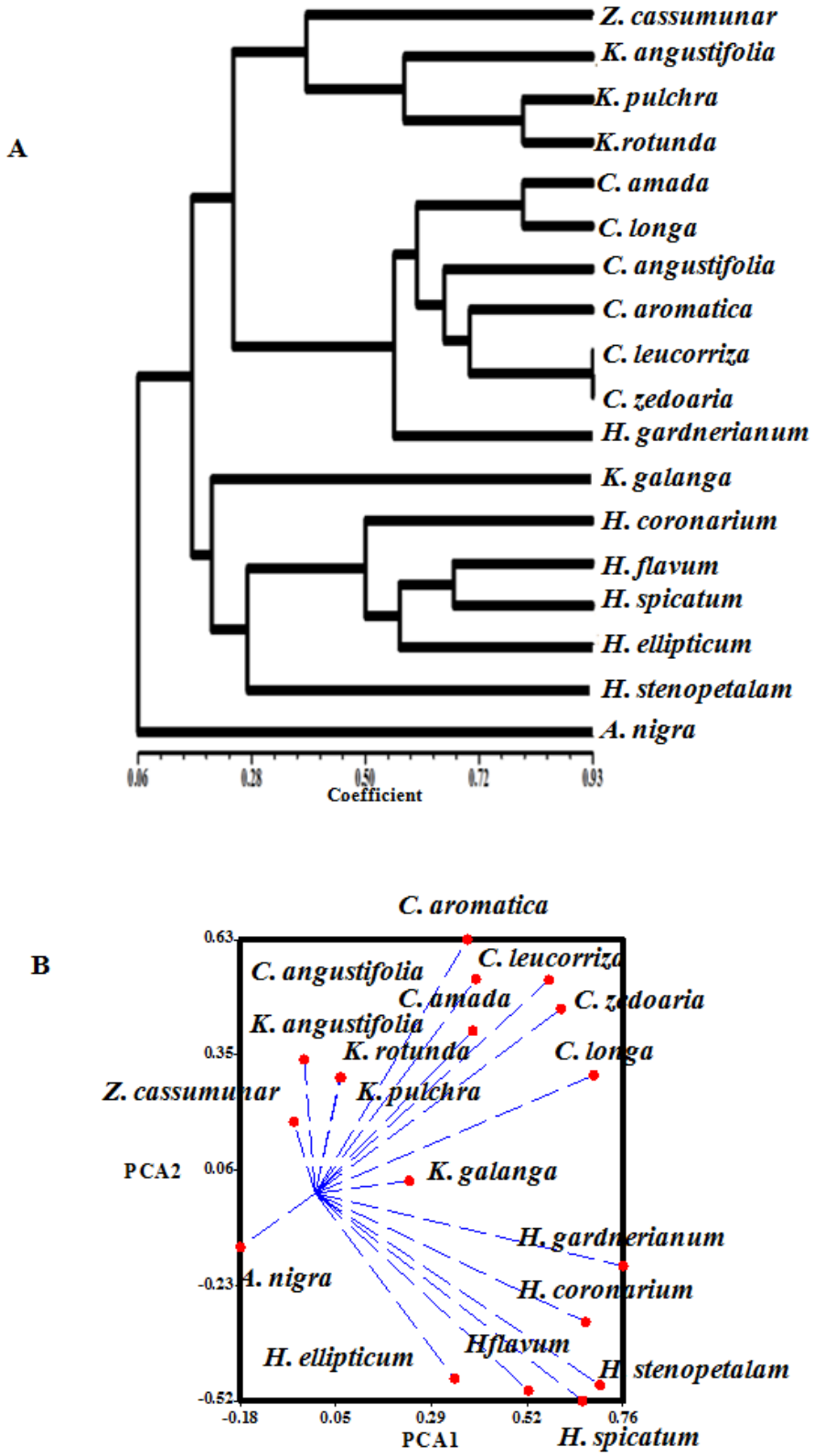

Fig 4D.1 Dendrogram for inter-generic genetic similarity of 18 species obtained from RAPD marker showing relationship within individuals. **A.** Dendrogram and **B.** PCA.

RAPD was able to group the species according to the out group. Cluster I consisted of species from *Hedychium* and cluster II consists of most of the species from *Curcuma* and *Kaempferia.* Cluster II has been grouped into two sub groups. Sub group I of cluster II consisted of species solely belonging to the genus *Curcuma,* sub group II consisted of the species from *Kaempferia* and *Zingiber.* So it was found that *Curcuma* and *Hedychium* formed compact clusters whereas *K. galanga* was falling within the genus *Hedychium.* The results of the cluster analysis were strongly supported by principal co-ordinate analysis (Fig. 4D.1).

Three major recognised clades (the *Curcuma* clade, the *Hedychium* clade, the *Camptandra* clade) were observed in the inter-generic study by previous workers (Kress et al 2002; Ngamriabsakul et al 2004). It has been shown that bootstrap support was higher for *Curcuma* clade (Ngamriabsakul et al 2004). The study closely follows the observation as *Curcuma* formed a tight cluster in RAPD based markers. In contrast, the sequence is very variable in *Kaempferia* which has the highest mutation rate among different genera (Ngamriabsakul et al 2004). Our study also revealed that *Kaempferia* is basically scattered between the *Curcuma* and *Hedychium* clusters.

4D.4 Conclusion

Two main sub clades were observed in Zingiberaceae, namely the *Curcuma* clade and the *Hedychium* clade. Our results showed paraphyletic origin of *Kaempferia* which is also supported by the previous studies. The data obtained from RAPD and ISSR markers will pave the way for all future studies.

CHAPTER 5

FLOW CYTOMETRIC STUDIES ON ZINGIBEROIDEAE AND OTHER SPECIES FROM THE FAMILY

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The flow cytometric studies of nuclear DNA content has been enumerated in this chapter.

CHAPTER 5A

PLANT FLOW CYTOMETRIC ANALYSIS - OPTIMISATION & STANDARDIZATION

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This chapter describes the optimisation of flow cytometric protocol for estimation of nuclear DNA content for a wide range of species.

PLANT FLOW CYTOMETRIC ANALYSIS - OPTIMISATION & STANDARDIZATION

5A.1 Introduction

Flow cytometry (FCM) using DNA - selective fluorochrome is now the widespread techniques for the measurement of nuclear DNA content in plants. The first application of flow cytometry in plant was the preparation of nuclei suspension of bean root tips fixed in alcohol acetic acid after enzymatic treatment of pectinase and pepsin (Heller 1973). Subsequent attempts to estimate the nuclear DNA content was severely impaired by the instrument cost (flow cytometer), difficulties in the preparation of suspension of intact cells and nuclei suitable for flow cytometry and other associated factors. Also, the labour intensive and skilled techniques further hampered the nuclear DNA content estimation which in turn delayed the progress of nuclear DNA content estimates. Early 1980's saw few publications on nuclear DNA content from intact cells and protoplasts. But the quality of histograms was not acceptable due to cell wall auto-fluorescence, blockage of the flow cell due to irregular cell dimensions (usage of intact cell), low permeability, and auto fluorescence of plasma membrane (usage of protoplasts) (Dolezel & Bartos 2005). This uneven cell cycle distribution was due to the 'off-centre' position of the nucleus (Galbraith 1990). The most popular strategic improvement of nuclear DNA content estimation has been shown by the analysis of intact nuclei, which might be released from protoplasts by lysis either in the presence of a detergent or in a hypotonic medium yielding histogram with the lowest possible coefficient of variation (Puite & Ten Broeke 1983).

In parallel, the development of protocols for DNA flow cytometry of mammalian cells observed a key discovery for the flow microfluorometric determination (lymphocytes) (Krishan 1975). Incubating cells in a hypotonic solution containing citric acid and propidium iodide (PI) resulted in disruption of the cell membrane and rapid staining of nuclear chromatin. DNA distribution histograms

generated from cells stained by this method are identical to those generated after fixation and RNase digestion. In contrast to some earlier described methods, the present technique is rapid (5 min of processing), requires a minimal amount of material, and avoids formation of cell clumps (Krishan 1975). In this work, modification of the hypotonic PI buffer for the plant DNA flow cytometric application by addition of detergent and RNase has been attempted for the Zingiberaceous species.

5A.2 Materials and methods

5A.2.1 Plant materials

Twenty six Zingiberaceae species (Table 5A.1) collected from their natural habitat of NE India and maintained in the green house conditions of Department of Biotechnology, IIT Guwahati were included in the current study. This includes 8 species of *Curcuma* and one cultivated variety (*C. domestica*), 6 species of *Hedychium*, 5 species of *Kaempferia*, cultivated variety of *Zingiber officinale* and the sacred variety of ginger from NE India (*Zingiber moran*, locally known as `moran ada´). Apart from this, four species often classified into separate but related genera were also included viz; *Globba bulbiflora*, *Boesenbergia rotunda*, *Costus speciosus* and *Alpinia nigra.*

Table 5A.1 Description of the plant materials studied

S. No.	Species Name	Collection point	Latitude and Longitude	Morphological Description	Habitat
1	*Curcuma zedoaria*	Darrang (Assam)	25°20´593´´N 92° 02´089´´E	Cone shaped tuber towards the end, yellowish, with strong smell	Shady, humid places
2	*Curcuma amada*	Amingaon (Assam)	26°18´824´´ N 91°67´ 850´´E	Light yellow, creeping, soft, strong smell like raw mango	Hilly slopes and moist grasslands
3	*Curcuma angustifolia*	Khasi hills (Shillong)	25°57´877´´ N 91°89´325´´E	White, tuber is hard with characteristic odour	Hilly moist slopes
4	*Curcuma domestica*	Kahikuchi (Assam)	26°35´855´´N 91° 13´ 134´´E	Rhizome is reddish in colour, larger tubers with strong smell	Shady, hot and humid places
5	*Curcuma longa*	Barpeta (Assam)	26° 31´712´´ N 91° 00´ 514´´ E	Yellowish, smaller tuber, with characteristic smell	Shady, moist places
6	*Curcuma leucorrhiza*	Amingaon(Assam)	26°18´824´´ N 91°67´ 850´´E	Green leaves , green midribs, yellowish rhizome with characteristic smell	Shady, moist places
7	*Curcuma* species	Avayapuri (Assam)	26°20´2´´N 90° 39´ 51´´E	Green leaves, no coloration in veins.	Shady, moist places
8	*Curcuma* species	Diglipara Forest (Assam)	26°26´83.9´´N, 90°29´62.5´´	Green leaves, with no coloration in veins.	Shady, moist places
9	*Curcuma aromatica*	Nagaon	26° 11´ N 92° 69´ 225´´E	Rhizome is hard, whitish in colour with strong smell	Shady, moist places
10	*Hedychium coronarium* J. Koenig	Guwahati (Assam)	26° 18´38´´ N 91°76´33´´ E	Oblong or oblong-lancealote leaves, large oblong imbricate bracts; white flower	Swamps and wet meadows
11	*Hedychium chrysoleucum* Hook.	Barpeta (Assam)	26°32´00´´ N 91°00´00´´E	Oblong or oblong-lancealote leaves, bracts*:* large oblong bracts, yellow flower	Humid and shaded areas
12	*Hedychium spicatum* Lodd.	Panbazar (Assam)	26°18´38´´ N 91°76´33° E	Oblong or oblong-lancealote leaves; large oblong bracts, white flower	From moderately to highly wet areas
13	*Hedychium* species	Margherita (Assam)	27°28´00´´N 95°68´00´´E	Oblong or oblong-lancealote leaves, large oblong bracts, white tinged with yellow flower.	Humid and shaded areas
14	*H. gardnerianum* Wall. ex Spreng.	Bomdila (Arunachal Pradesh)	27°25´00´´ N 92°40´00´´ E	Oblong, white pulverulent beneath leaves, large oblong bracts, bright lemon yellow flowers	Windward hilly slopes
15	*Hedychium ellipticum*	Silchar (Assam)	25°27´901´´ N 92°13´560´´E	Leaves*:* lanceolate ; Bracts*:* oblong; Flowers*:* small bright crimson	Hilly slope
16	*Kaempferia galanga* L.	Khetri (Assam)	26° 11´ 49´´ N 92°09´ 10´´E	Slightly dark green shiny, wavy margin, acute apex - dorsal rough ventral, light green wavy margin- ventral	Humid and shaded areas

S. No.	Species Name	Collection point	Latitude and Longitude	Morphological Description	Habitat
17	*Kaempferia angustifolia* Roxb.	Jalukbari (Assam)	26° 14´ 40´´N 91° 67´ 12´´ E	Dark green velvety texture, acute apex - dorsal Rough light green- ventral	Humid and shaded areas
18	*Kaempferia pulchra* Ridl.	Jalukbari (Assam)	26° 14´ 40´´N 91° 67´ 12´´ E	Dark green, velvety texture, patterned coloration (green) Slightly wavy margin, large in size, acute apex, green midrib – dorsal. Purple, parallel venation, hairy, velvety texture- ventral	Humid and shaded areas
19	*Kaempferia pulchra* Ridl. 1	Ultapani Reserve Forest (Assam)	26° 77´ 591´´ N 90° 30´4215´´E	Dark green, velvety texture, patterned coloration (green) Slightly wavy margin, large in size, acute apex, green midrib – dorsal. Purple, parallel venation, hairy, velvety texture - ventral	Humid and shaded areas
20	*Kaempferia rotunda* Blanco.	NEDFi (Assam)	26° 20´ 06´´N 92° 93´ 75´´E	Parallel venation, patterned coloration (green), regular margin, shiny – dorsal Purple, parallel venation, hairy, velvety texture - ventral	Humid and shaded areas
21	*Globba bulbiflora*	Ultapani Reserve Forest (Assam)	26° 77´ 591´´ N 90° 30´4215´´E	It has upright flower spike. It is propagated by division and by the adventitious corms.	Humid and shaded areas
22	*Boesenbergia rotunda*	Ultapani Reserve Forest (Assam)	26° 77´ 591´´ N 90° 30´4210´´E	Its leaves are broad and light green while the leaf sheath is red. Rhizome is basically small globular shaped and central subterraneous	Humid and shaded areas
23	*Zingiber officinale*	Guwahati (Assam)	26° 46' 33.2754" N 90° 18' 15.1734" E	Leaves are clustered at the base or scattered along a distinct stem, alternate, sessile or petiolate, usually caudate, acuminate, linear and narrow	Humid and shaded area
24	*Zingiber moran*	Sivasagar (Assam)	26° 46' 33.2754" N 90° 18' 15.1734"E	Leaves are clustered at the base or scattered along a distinct stem, alternate, sessile or petiolate, usually caudate, acuminate, linear and narrow	Humid and shaded area
25	*Costus speciosus*	Seijosa (Arunachal Pradesh)	26° 46' 33.2754" N 90° 18' 15.1734"E	Leaves are oblong, spirally arranged, silky-pubescent beneath; sheaths coriaceous	Humid and shaded area
26	*Alpinia nigra*	Sivasagar (Assam)	26° 46' 32.2752" N 90° 18' 15.1734"E	the presence of a rhizome, simple, wide-brim leaves protected by showy bracts, and terminal inflorescences	From moderately to highly wet areas.

5A.2.2 Standards for flow cytometric estimation of nuclear DNA content

Seeds of *Oryza sativa* 'IR36' (2C = 1.01 pg, Price and Johnston, 1996) were obtained from the appropriate USDA germplasm collections (GRIN: http://www.ars-grin.gov/npgs/). *Solanum lycopersicum* cv. Stupicke (2C= 1.96 pg, Dolezel et al 1992), *Zea mays* CE-777 (2C = 5.43 pg, Lysak & Dolezel 1998) and *Pisum sativum* cv. Citrad (2C = 9.09 pg, Dolezel 1998) recommended standard reference species for estimating DNA content were gently supplied by Dr. J. Dolezel. *Solanum lycopersicum* cv. Stupicke was used as the primary reference standard whereas *Oryza sativa* 'IR36', *Zea mays* CE-777, *Pisum sativum* cv. Citrad were used for the species with higher and lower nuclear DNA contents.

5A.2.3 Sample preparation and isolation of nuclei

The youngest fully developed leaf sheath was used for the preparation of the suspension of intact nuclei. The leaves (1 cm^2) were chopped with a razor blade in modified 1.0 mL of propidium iodide/hypotonic Citrate Method of Awtar Krishan (Krishan 1975) containing 0.1 % w/v sodium citrate (Sigma-Aldrich, cat. no. S4651), 0.3 % v/v detergent IGEPAL CA- 630 (Sigma-Aldrich, cat. no. I3021). Staining was done with 25 mg L^{-1} PI (Sigma-Aldrich, cat. no. P4170) in a solution containing DNase - free RNase A (2 mg/ml) (Sigma-Aldrich, cat. no. P4875). The suspension of nuclei was filtered through 30 µm nylon mesh (Swedesboro, NJ) and was taken for flow cytometric analysis.

5A.2.4 Use of filter mesh

The effect of filter mesh on the outcome of flow cytometric protocol was checked by using different dimension of filter mesh. The filter mesh used was 30, 50 and 100 microns respectively (CellTrics® Partec, Germany).

5A.2.5 Effect of detergent concentration

The hypotonic buffer contained 0.03% (v/v) detergent concentration (IGEPAL CA- 630). The detergent (IGEPAL CA- 630) concentration was increased from 0.03% to 0.3% (v/v) for release of the nuclei from the cell.

5A.2.6 Test for inhibitors

Zingiberaceae extracts were tested for unidentified compounds that reduce PI fluorescence of standard nuclei as follows. Nuclei were released from an approximate 40 mm x 20 mm leaf and one-half of a standard leaf that were simultaneously processed (co-chopped) and stained with PI (sample A). Sample B consisted of PI-stained nuclei from the independently processed and stained other half of the standard leaf used in sample A. After staining for 1 h, samples A and B were individually measured for mean PI fluorescence, after which they were mixed and measured up to 120 min. The experiment was repeated three times. Reduced fluorescence of nuclei from standard leaves simultaneously processed with Zingiberaceae leaves when compared to nuclei from independently processed standard leaves gave evidence of Zingiberaceae inhibitors.

5A.2.7 Bright field and fluorescence microscopy

Nuclear suspensions were assessed in a Nikon Eclipse 80i fluorescence microscope (Nikon Corporation Nikon Instech Co., Kanagawa, Japan) using the G-2A filter cube. Digital photographs were taken using a Leica DC 200 digital camera (Leica Microsystems AG, Wetzlar, Germany).

5A.2.8Flow cytometric analysis

Estimation of nuclear DNA content was performed with BD FACS Calibur flow cytometer (BD Biosciences, New Delhi, India). Samples stained with PI were excited with a 15-mW argon ion laser at 488-nm. PI fluorescence was collected through a 645-nm dichroic long-pass filter and a 620-nm band-pass filter. The instruments settings i.e., voltage and gain were kept constant throughout the experiment. Three dot plots and one histogram plot ware generated. Three dot plots were: (i) FSC vs. SSC to understand the size and granularity of the nucleus; (ii) SSC vs FL2A to spot intense fluorescence regions due to nuclei and to eliminate background fluorescence by drawing electronically a gate region around the signals due to intact nuclei; (iii) FL2A vs. FL2W to discriminate between singlets and doublets. Frequency vs. FL histogram was generated to compare the mean position of the sample peaks relative to the internal standard. For each sample

at least 10,000 nuclei were analyzed at a rate of 20-50 nuclei per seconds. Test sample and standards were co-chopped in the internal standardization. The samples of standard plant nuclei and Zingiberaceous plants were chopped separately then mixed together just before acquiring in pseudo-internal standardization. The nuclear DNA content was estimated by comparing the mean fluorescence intensity of nuclei of the sample material with that of the reference standard and obtained by multiplying the nuclear DNA content of standard species by the ratio of their fluorescence intensities. The results were acquired using BD Cell Quest Pro software (version 6.0, BD Biosciences). The resulting histograms were analyzed using FlowJo v. 7.6.5 (FlowJo, TreeStar Inc, Ashland, OR) for estimating mean fluorescent intensity (MFI), co-efficient of variation (CV). A suitable gating procedure was followed to resolve the data and to measure the parameters only for intact nuclei in a heterogeneous population. Auto fluorescence due to chloroplast was eliminated from analysis by selecting a region on fluorescence in FITC channel and PI fluorescence profile. To reduce the level of debris and disintegrated nuclei, the nuclei were gated in PI fluorescence channel vs. SSC dot plots. In this diagram the nuclei can clearly be identified by their defined fluorescence intensity/scattered light pattern. Doublets and clumps were eliminated by gating on fluorescence width and fluorescence area profiles. In this histogram, an “interest zone” was defined such that only single intact nuclei were included in the fluorescence histogram. The step-by-step gating procedure followed in this study is described in Fig5a.1. The nuclear DNA content (picograms) was converted to base pairs by considering that 1 pg of DNA corresponds to 978Mb (Dolezel et al 2003). The analysis was repeated if the coefficient of variation of the sample was >5.0.

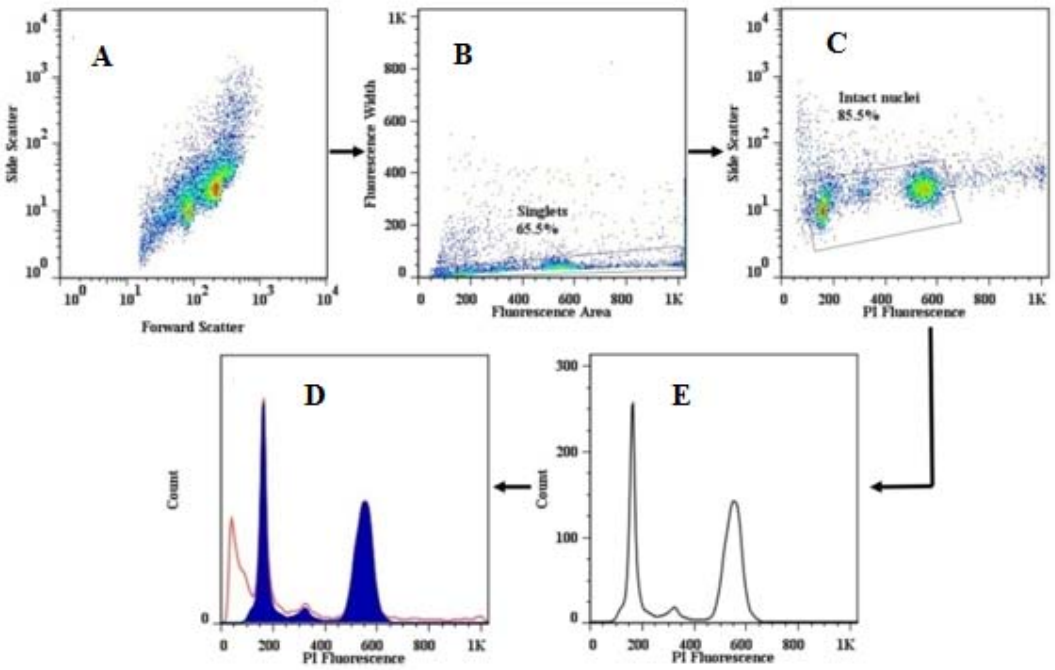

Fig 5A.1 A step-by-step gating regime followed for Zingiberaceae plants to resolve our flow cytometric histograms. **A.** Side scatter vs. Forward scatter profile **B.** Selecting an interest zone on PI fluorescence width vs. area profile to eliminate doublets and other clumps. **C.** Selecting an interest zone on SS log vs. PI fluorescence profile to eliminate debris and disintegrated nuclei. **D.** Resolved histogram showing G0/G1 and G2/M peaks of *Pongamia* and reference standard peak. **E.** Resolved histogram showing the debris removed via the gating regime (red)

5A.3 Results and discussion

5A.3.1 Effect of filter mesh

The usage of unfiltered and the filtered suspension (100 and 30 micron filter mesh) of processed material in PI/ micron filter mesh had no significant difference in the nuclei observed for the standard plant (*P. sativum*) (Fig 5a.2 A, D and H). However the scatter plot represented appearance of tight cluster in the filtered material through 30 micron filter mesh (Fig 5a.2 B, E and I). So usage of 30 micron filter mesh was optimized for the all standard plant materials for the subsequent flow cytometric analysis. The microscopic view of the unfiltered suspension of the chopped material of the Zingeberaceous species provided appearance of the cells but no free nuclei. Therefore, this was not suitable for flow cytometric analysis as this could damage the flow cell. Subsequently the usage of the 100 micron and 30 micron filter mesh resulted in the significant decrease in the number of cells in the bright field microscopic analysis (Fig 5A.2 C, F &J). The decrease in the intensity of cell cluster was also seen in the scatter plot of the analysis (Fig 5A.2 G & K).

5A.3.2 Effect of detergent concentration

The hypotonic PI [Sodium citrate 0.1% (w/v), 0.03% (v/v) NP 40, 25 µg/ml and 0.5 mg/ ml] was able to release the nuclei from all the standard species. The prolonged incubation at different temperatures (37°C and 65°C) could not break open the cells. The release of nuclei was standardized by increasing the NP 40 concentration gradually from 0.03% to 0.3% (Fig 5a.3). The release of nuclei from the cells in other Zingiberaceous speciesis shown [*C. longa, C. amada, H. coronarium, K. galanga K. angustifolia* (Fig 5A.4)].

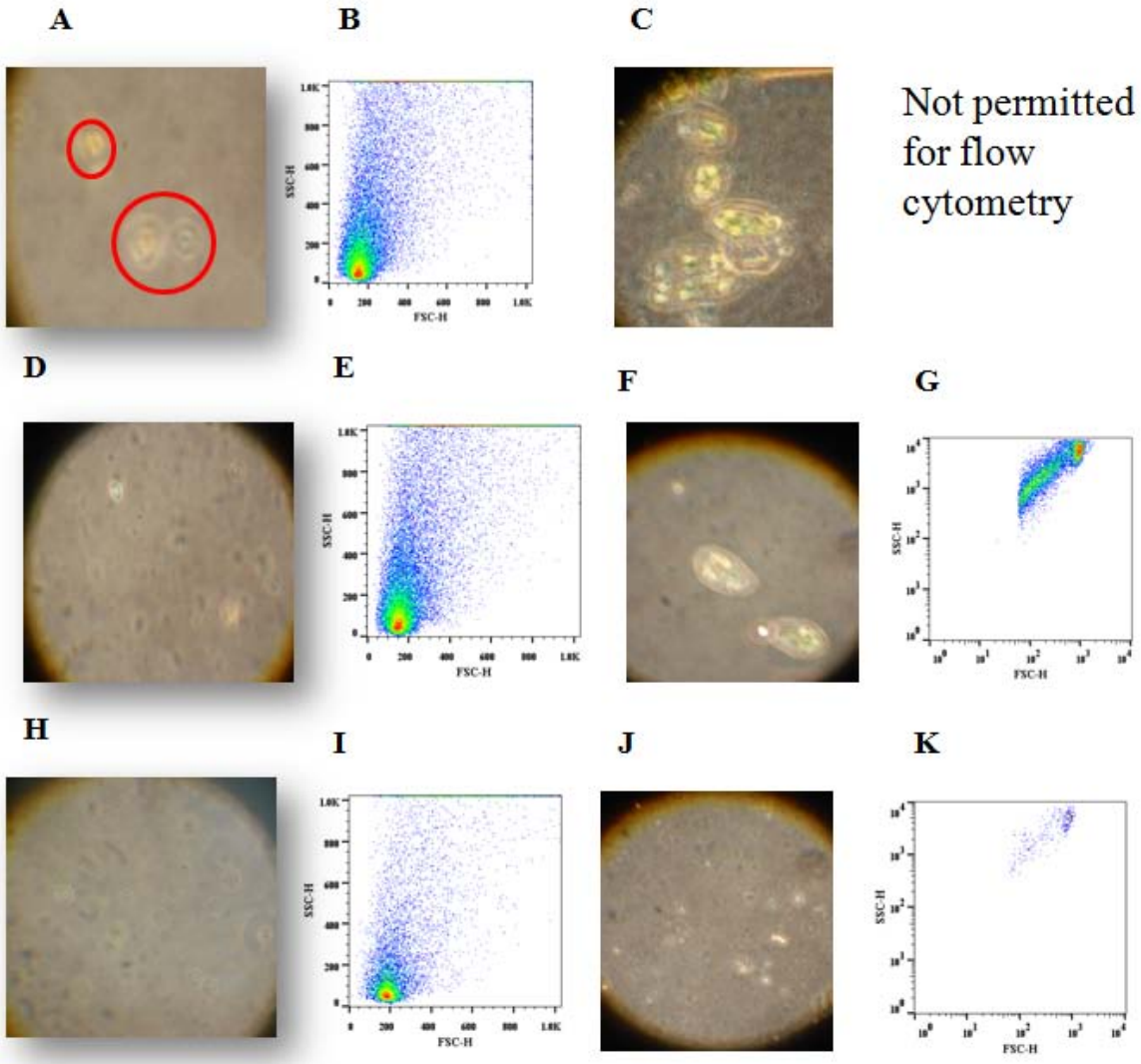

Fig 5A.2 Bright field and flow cytometric comparison. Bright field microscopic image and scatter plot of standard (*P. sativum*) and test (*C. zedoaria*) of the plant samples chopped in nuclear isolation buffer (Krishan 1975) before filtration through nylon mesh [**A.** bright field (40X) and **B.** scatter plot of *P. sativum* **C.** bright field (40X) of *C. zedoaria*] after filtration through 100 micron nylon mesh [**D.** bright field (40X) and **E.** scatter plot of *P. sativum* **F.** bright field (40X) of *C. zedoaria* **G.** scatter plot of *C. zedoaria*], 30 micron nylon mesh [**H.** bright field (40X) and **I.** scatter plot of *P. sativum* **J.** bright field (40X) of *C. zedoaria* **K.** scatter plot of *C. zedoaria*].

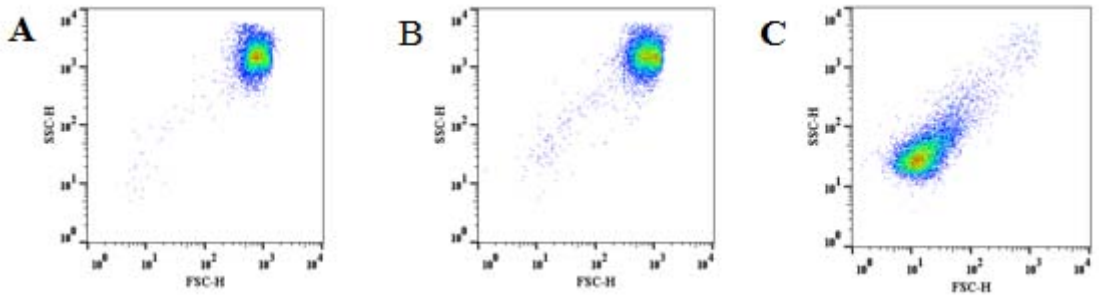

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Fig 5A.3 Effect of detergent concentration on the lysis of cells of *C. zedoaria*. **A.** Nuclear isolation buffer (Krishan 1975) containing 0.03% (v/v) NP 40. **B.** Nuclear isolation buffer with 0.10% (v/v) NP 40. **C.** Nuclear isolation buffer with 0.3% (v/v) NP 40.

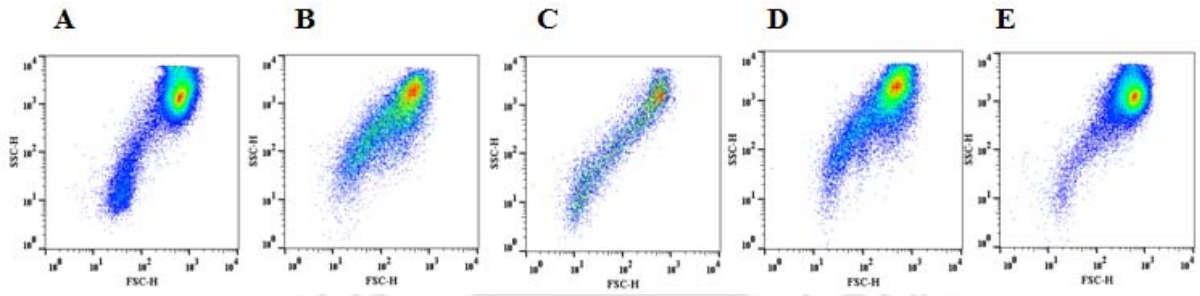

Fig 5A.4 Differential release of nuclei in 0.3% of detergent NP 40. **A.** *C. longa* **B.** *C. amada*. **C.** *H. coronarium* **D.** *K. galanga* **E.** *K. angustifolia*

5A.3.3 Microscopic and flow cytometric analysis: external standardization

Microscopic analysis revealed the size and intensity difference between the *Z. mays* CE777 (2C =5.43 pg) with the *C. zedoaria* nuclei. For *Z. mays* CE777 free nuclei were observed and for the *C. zedoaria* nuclei were small in the dimension and some of the nuclei were found to be attached to the cell and some were freely suspending in the nuclei isolation buffer (Fig 5A.5). The appearance of bright nuclear fluorescence associated with a background was found due to the cellular fluorescence (Fig 5A.5).

5A.3.4 Test for inhibitors

Zingiberaceae leaves may contain substance(s) that inhibit PI fluorescence of standard leaf nuclei. Price et al (2000) experiment was conducted to test for the presence of inhibitors. No peak shifting of the standard nuclei was observed after mixing the independently processed standard (*P. sativum*) nuclei and co-processed test plant (*K. angustifolia*) and standard plant (*P. sativum*). This confirms that Zingiberaceae plant contains no inhibitors (Fig 5A.6).

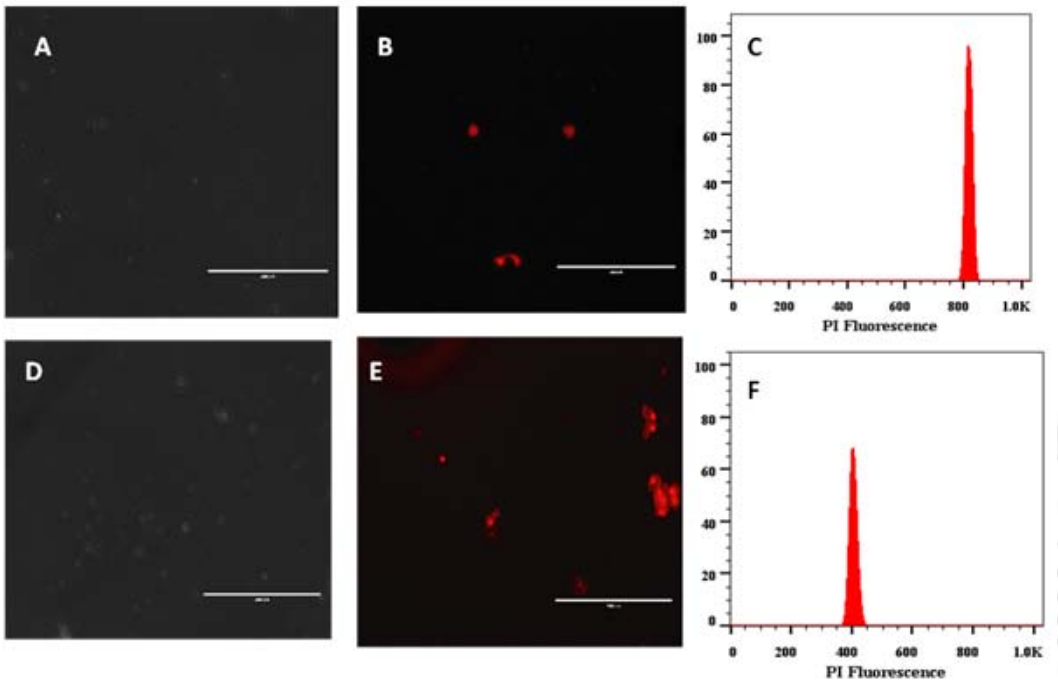

Fig 5A.5 Bright field and fluorescence microscopic photograph of *Z. mays* and *C. amada*. **A** and **D** are the bright field microscopy image of the nuclear suspension prepared in hypotonic PI of *Z. mays* and *C. amada*, respectively. **B** and **E** are the fluorescence microscopic image of the nuclear suspension prepared in hypotonic PI of *Z. mays* and *C. amada* respectively. **C** and **F** histogram of median fluorescence intensity of the nuclear suspension prepared in hypotonic PI of *Z. mays* and *C. amada* respectively.

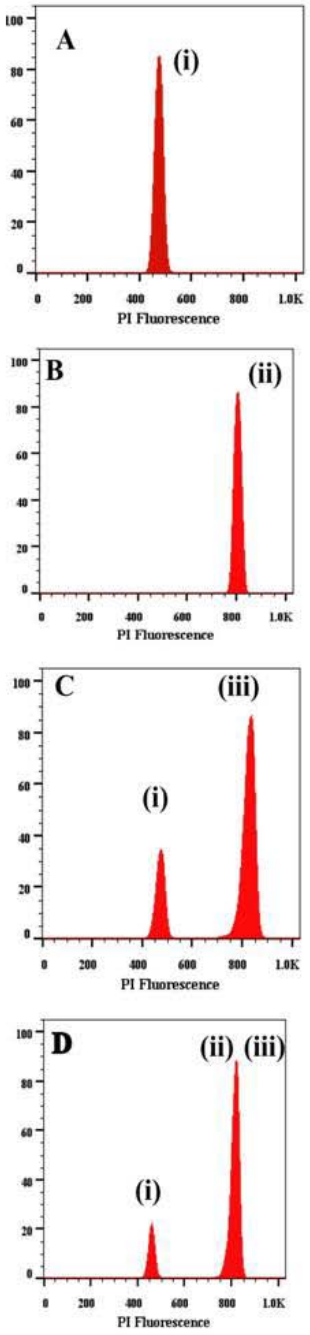

Fig 5A.6 Test for inhibitors. Histogram of PI stained nuclei from simultaneously processed *K. angustifolia* (i) and standard (ii) leaves to which PI stained nuclei from independently processed standard leaves (iii) were added **A.** *P. sativum* (external standardization) MFI: 835 (*P. sativum*) **B.** *K. angustifolia* (external standardization) **C.** *K. angustifolia* and *P. sativum* (internal standardization) **D.** *K. angustifolia* and *P. sativum* (internal standardization) and *P. sativum* external standardization (mixed).

5A.4 Conclusion

From this chapter, it could be concluded that the flow cytometric protocol was standardized for the standards and the test plants. The optimised buffer was effective for a wide range of Zingiberoideae species. The inhibitors of the Zingiberaceae plants were not observed. This might be due to the absence of secondary metabolites present in the leaves of Zingiberoideae plants. The inhibition was minimum so the user could operate the estimation of C-value with precision.

CHAPTER 5B

DETERMINATION OF OPTIMUM TISSUE TYPES FOR FLOW CYTOMETRIC STUDY

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This chapter describes the selection of plant tissue for flow cytometric studies of nuclear DNA content.

DETERMINATION OF OPTIMUM TISSUE TYPES FOR FLOW CYTOMETRIC STUDY

5B.1 Introduction

Eukaryotic organisms are defined by the presence of a nucleus, which encloses the chromosomal DNA, and is characterized by its DNA content (C-value). For many eukaryotes, the nuclei of somatic cells contain 2C DNA amount, and the growing cells participatein a simple mitotic cell cycle in which four temporally linked phases, G1, S, G2 and M, serve to separate the processes of DNA duplication (S-phase) from chromosomal segregation (M-phase). Monosomatic tissues containing mitotically active cells therefore are characterized by cells having nuclear DNA contents ranging from 2C to 4C depending on the position of the cells within the cell cycle. In polysomatic tissues, the situation is complicated by the occurrence of an alternative cell cycle, termed endoreduplication, in which successive S-phases are not followed by M-phases. This produces uninucleate cells having multiplicative DNA contents (2^n C, where n =1,2,3..., for most sources of somatic cells, and $3 \times 2^{n-1}$ C for the endoreduplicated endosperm derived from triploid progenitor cells). Polysomaty is particularly common in higher plants (Joubès & Chevalier 2000); for some species, such as *A. thaliana*, it is encountered throughout the mature tissues of the organism (Galbraith 1999) while in others it is restricted to specific tissues (Barow & Meister 2003)

The functional significance of the state of the nuclear C-value at which DNA synthesis arrests remains obscure, inpart due to a lack of facile and precise methods for identifying its occurrence as a function of specific cell types. It is clear that, in the analysis of developmental gene expression and the cell biology underlying its regulation, the nuclear C-value represents an important parameter reflecting both the cell cycle status of the cell within which the nucleus is located, as well as the participation of the cells of polysomatic tissues within cycles of endo-reduplication.

Conversely, the regulated arrest of the cell at specific nuclear C-values reflects the activities of regulatory mechanisms about which we know very little.

The variation of nuclear DNA content with tissue types (mitotically active cells, non dividing cells along with flower) of Zingiberaceae has not been documented. The current work corroborates variation of the nuclear DNA content in different tissue types.

5B.2 Materials and methods

5B.2.1 Plant materials

To evaluate the suitable plant tissue for flow cytometric estimation of Zingiberaceae species, 10 different tissue types of five species of Zingiberoideae, representing three different genera, viz., *C. zedoaria*, *C. amada*, *C. angustifolia*, *K. pulchra* and *Boesenbergia rotunda*were collected (Fig 5B.1). Based on the flower availability *C. specious* (Costaceae) was also included.The different tissue types were leaf, root tip, rhizome, stem, style, stigma, staminode, gynoecium, calyx and corolla (Fig 5B.2).

5B.2.2 Sample preparation and flow cytometric estimation

The details of the sample preparations and flow cytometric estimation discussed in section 5a.2.3 and 5a.2.8, respectively.

5B.3 Results and discussion

Nuclear DNA contents of Zingiberaceae species were presented as picograms against four different standards and the variability of 2C-values among different tissues from single species was tested using one-way ANOVA (Table 5B.1). Our results showed that 2C-values of the ten tissues from a single Zingiberaceous plants had significant differences among each other (P = 0.05) by internal standardization. The estimation of 2C-values, taking *C. zedoaria* for example, were 3.13± 0.01, 3.12± 0.03, 3.20 ± 0.02, 2.51± 0.04, 2.85± 0.04, 2.59± 0.04, 2.06± 0.03 and 3.07± 0.02 pg for leaf, root tip, rhizome, stem, style, gynoecium, calyx and corolla respectively (P = 0.0.00 < 0.05) Although nuclear DNA content of *C. zedoaria* leaf, root and corolla resulted in non-significant variation (P = 0.16>0.05).

Rhizome displayed a different order of nuclear DNA content from that of other parts of the plants. Stem and gynoecium had no statistical difference in nuclear DNA amounts (P= 0.217 > 0.05).

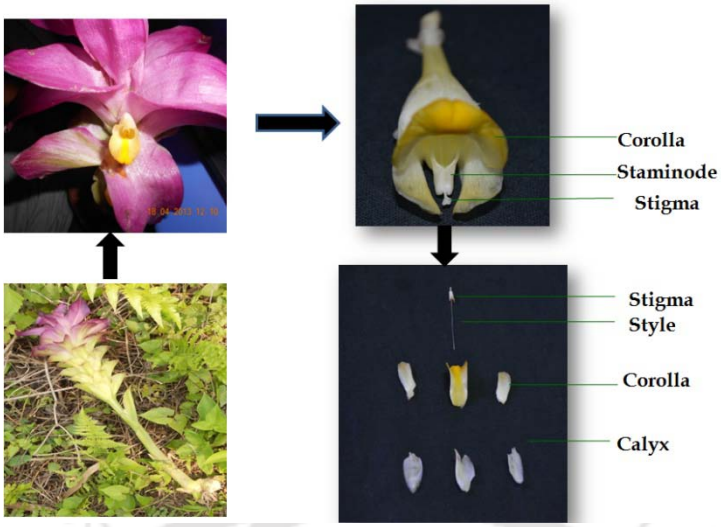

Fig 5B.1 Anatomy of *C. zedoaria* flower

Fig 5B.2 Inflorescence/ flower along with floral dissection (five different part of the flower) for the study of tissue specific variation in the family Zingiberaceae. (a) *C. zedoaraia*, (b) *C. amada*, (c) *C. angistifolia*, (d) *B. rotunda* (e) *K. pulchra* (f) *C. speciosus.* (i) Stigma, (ii) Style, (iii) Ovary, (iv) Corolla , (v) Calyx.

The highest and the lowest nuclear DNA content of *C. zedoaria* were 3.20 pg (rhizome) and 2.06 pg (calyx) with the 1.55 fold variation. Tukey's test for *S. lycopersicum* has been shown in Table 5B.2. The experimental findings of nuclear DNA content of *C. amada* showed that significant variation in DNA content of various tissue types (P = 0.00 <0.05). Nuclear DNA content of *C. amada* (leaf, root, rhizome) and the gynoecium resulted in significant variation (P = 0.00 <0.05). But the nuclear DNA content of style, stigma, staminodes and calyx were on the higher side (2C=2.06 pg) and resulted in non-significant variation (P=0.07> 0.05). The maximum and minimum value of the nuclear DNA content of *C. amada* with their tissue types was observed to be 2.08 pg (staminodes) and 1.80 pg (calyx). The fold variation within the different tissue types of *C. amada* is reported to be 1.54 fold. For 7 different tissue types of *C. angustifolia,* significant variation in nuclear DNA content was observed (P=0.00 < 005). The interesting phenomenon of the nuclear DNA content variation study was the appearance of three order magnitude among the vegetative part [leaf and rhizome; 2C = 2.46 pg (P = 0.50 > 0.05)], floral part I [staminode and stigma, 2C = 2.07 pg (P = 0.50 > 0.05)]; floral part II [style, corolla and gynoecium, 2C = 3.07 pg (P = 0.50 > 0.05)]. So it was observed that nuclear DNA content of the reproductive part of *C. angustifolia* was different from the vegetative parts (leaf and roots). Nuclear DNA content variation study of *C. angustifolia* revealed maximum and minimum nuclear DNA content of 3.17 pg (style) and 2.06 pg (stigma) in 7 different tissues with the fold variation of 1.54. The nuclear DNA content from eight different tissue types of *B. rotunda* resulted in significant variation (P = 0.00 < 0.05). The vegetative parts (leaf, root, rhizome) resulted in significant variation in nuclear DNA content (P = 0.49 > 0.05). The floral parts (style, stigma, gynoecium, calyx and corolla) of *B. rotunda* showed statistically significant variation in the nuclear DNA content (P = 0.00 < 0.05). The highest and the lowest nuclear DNA content was observed in stigma (2C = 8.39 pg, *P. sativum* as standard) and style (2C = 5.80 pg, *P. sativum* as standard) respectively, with the 1.45 fold variation. Eight tissue types of *K. pulchra* resulted in statistically significant variation in nuclear DNA content (P = 0.00 < 0.05). Although the vegetative part of *K. pulchra* (leaf, root, rhizome and stem) yielded statistically similar nuclear DNA content (P = 0.15 > 0.05). The reproductive part of the plant resulted in significant variation (P =

0.00 < 0.5), partly due to the higher nuclear DNA content of calyx of *K. pulchra* (2C = 3.30 pg *Z. mays* as standard) compared to the other parts (2C = 2.97 pg for stigma, style, gynoecium and corolla using as standard). The maximum and the minimum value of the nuclear DNA content of *K. pulchra* was experimentally found to be 3.44 pg (stem; *Z. mays* as standard) and 2.94 pg (style; *Z. mays* as standard) respectively. The fold variation was reported to be 1.17 fold in *K. pulchra*. *C. speciosus* showed the highest discrepancy in the nuclear DNA amounts in seven different tissue types (P = 0.00 < 0.05). Tissue types were segregated in three different parts viz; dividing cells (leaf, root tips; 2C = 3.38 pg using *Z. mays* as standard), non-dividing cells (rhizomes and stem; 2C = 2.26 pg using *Z. mays* as standard) and floral parts (stigma, style and gynoecium; 2C = 2.18 pg taking *Z. mays* as standard), resulted in significant variation in the nuclear DNA content (P = 0.49 > 0.05). The highest and the lowest nuclear DNA content were estimated to be 3.55 pg (leaf) and 1.79 pg (stigma), respectively with 1.98 fold variation.

In general, reproductive parts of the plant represent a different order of nuclear DNA content. The DNA content variation within the same plant is also reported (Meagher & Costich 1994, Zhang et al 2005). The genome size variation with the flower type (male flower and female flower) and flower dimension has been already documented (Meagher & Costich 1994). The tissue type specific variation in nuclear DNA content in dividing and non-dividing tissues has been established (Zhang et al 2005). Extensive variation in genomesize was perceived to be much greater than necessary to account for the corresponding range of organismic complexity, and this perception gave rise to the C-value enigma (Gregory 2001). However, as a result of modern genomic analysis, such variation is now knownto be coupled with genome structure and organization. Specifically, much of the variation in DNA content observed between and within taxa is attributable to different categories of repetitive DNA, some of which have been shown to have an impact on patterns of gene expression. .*A thaliana* has a high level of endoploidy in nearly all parts of the plant (cotyledon, root, lower leaf stalk, lower leaf, upper stem, upper leaf, flower stalk, sepal, petal (Galbraith et al 1991).

Tale 5B.1 Comparisons of nuclear DNA amount (2C, pg) estimated using flow cytometry in different tissues of the six species of Zingiberaceae

S. No	Plant part	Internal standards	Zingiberaceous Species					
			Curcuma zedoaria	*Curcuma amada*	*Curcuma angustifolia*	*Boesenbergia rotunda*	*Kaempferia pulchera*	*Costus speciosus*
1	Leaf	*O. sativa*	2.93± 0.02	1.68± 0.01	1.97± 0.02	9.29± 0.02	3.42± 0.02	-
		S. lycopersicum	3.13 ± 0.01	1.92± 0.02	2.41± 0.04	8.93± 0.02	3.55± 0.04	-
		Z. mays	2.63± 0.01	2.06± 0.03	2.25± 0.01	9.02± 0.05	3.20± 0.02	2.36± 0.02
		P. sativum	-	-	-	7.95± 0.04	3.74± 0.04	3.25± 0.03
2	Root tips	*O. sativa*	2.91± 0.02	1.68± 0.02	2.04± 0.04	8.58± 0.01	3.55± 0.05	-
		S. lycopersicum	3.12± 0.03	1.92± 0.04	2.51± 0.02	8.24± 0.04	3.68± 0.02	-
		Z. mays	2.62± 0.04	2.06± 0.01	2.33± 0.01	8.33± 0.05	3.32± 0.05	3.21± 0.04
		P. sativum	-	-	-	7.34± 0.02	3.88± 0.05	4.4± 0.012
3	Rhizome	*O. sativa*	2.99± 0.03	1.54± 0.04	-	8.72± 0.04	3.58± 0.02	-
		S. lycopersicum	3.20± 0.02	1.75± 0.01	-	8.38± 0.05	3.71± 0.01	-
		Z. mays	2.69± 0.04	1.88± 0.02	-	8.47± 0.02	3.35± 0.05	2.14± 0.02
		P. sativum	-	-	-	7.47± 0.05	3.91± 0.04	2.95± 0.04
4	Stem	*O. sativa*	2.35± 0.03	-	-	-	3.68± 0.02	-
		S. lycopersicum	2.51± 0.04	-	-	-	3.81± 0.05	-
		Z. mays	2.11± 0.02	-	-	-	3.44± 0.02	2.38± 0.02
		P. sativum	-	-	-	-	4.02± 0.05	3.27± 0.04
5	Stigma	*O. sativa*	-	1.61± 0.01	1.69± 0.02	9.80± 0.05	3.18± 0.03	-
		S. lycopersicum	-	1.83± 0.02	2.06± 0.01	9.42± 0.02	3.30± 0.03	-
		Z. mays	-	1.97± 0.04	1.93± 0.04	9.52± 0.05	2.98± 0.03	1.79± 0.04
		P. sativum	-	-	-	8.39± 0.04	3.48± 0.03	2.47± 0.01
6	Style	*O. sativa*	2.65± 0.02	1.70± 0.03	2.59± 0.01	8.01± 0.02	3.14± 0.05	-
		S. lycopersicum	2.85± 0.04	2.08± 0.03	3.17± 0.04	7.70± 0.03	3.26 ± 0.04	-
		Z. mays	2.57± 0.03	1.94± 0.04	2.95± 0.01	7.78± 0.03	2.94± 0.04	2.39± 0.05
		P. sativum	-	-	-	6.86± 0.02	3.43± 0.02	3.28± 0.04
7	Gynoceum	*O. sativa*	2.41± 0.02	1.66± 0.04	2.57± 0.04	8.03± 0.04	3.18± 0.04	-
		S. lycopersicum	2.59± 0.04	2.03± 0.02	2.94± 0.05	7.71± 0.03	3.30± 0.04	-
		Z. mays	2.33± 0.03	1.92± 0.04	3.15± 0.02	7.79± 0.03	2.98± 0.03	2.38± 0.05
		P. sativum	-	-		6.87± 0.03	3.48± 0.05	3.27± 0.02
8	Calyx	*O. sativa*	1.92± 0.02	1.46± 0.03	-	8.24± 0.04	3.54± 0.04	
		S. lycopersicum	2.06± 0.03	1.80± 0.02	-	7.92± 0.02	3.66± 0.02	
		Z. mays	1.86± 0.02	1.67± 0.02	-	8.00± 0.02	3.30± 0.01	
		P. sativum	-	-	-	7.05± 0.01	3.85± 0.01	
9	Corolla	*O. sativa*	2.86± 0.04	1.68± 0.01	2.53± 0.02	6.78± 0.04	3.18± 0.04	
		S. lycopersicum	3.07± 0.02	2.06± 0.01	3.10± 0.01	6.51± 0.01	3.30± 0.01	
		Z. mays	2.76± 0.01	1.92± 0.02	2.89± 0.04	6.58± 0.02	2.98± 0.01	
		P. sativum	-	-	-	5.80± 0.04	3.48± 0.04	
10	Staminodes	*O. sativa*	-	1.70± 0.01	1.87± 0.02	-		
		S. lycopersicum	-	2.08± 0.02	2.29± 0.01	-		
		Z. mays	-	1.94± 0.02	2.13± 0.04	-		
		P. sativum	-	-	-	-		

Tale 5B.2 Statistical grouping of the nuclear DNA amounts of *C. zedoaria* during internal standardization

		N	Subset for alpha = 0.05						
			1	2	3	4	5	6	7
Tukey HSD[a]	calyx	3	2.0600						
	stem	3		2.5200					
	gynoecium	3		2.5667					
	style	3			2.8500				
	corolla	3				3.0700			
	root tip	3				3.1167			
	leaf	3				3.1200			
	rhizome	3					3.1933		
	Sig.		1.000	.217	1.000	.160	1.000		

**S. lycopersicum* used as internal standard

5B.4 Conclusion

It could be said that nuclear DNA content across the studied tissue types of the Zingiberaceous species were not uniform. Zingiberaceous species being perennial in nature accounts variability in nuclear DNA content not only at the inter species level but also in the different tissue types of the same species. In the current study the highest variation in the different tissue types was observed in *C. speciosus* and the lowest variation in nuclear DNA content was observed in *C. amada*. This study opens the light on the study of the transposable DNA in different tissue types. This raises the concept of endoploidy in the studied Zingiberaceous species.

CHAPTER 5C

INTRA-SPECIES VARIATION IN NUCLEAR DNA CONTENT OF TURMERIC VARIETY OF NORTH EAST INDIA

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This chapter gives information of the nuclear DNA content variation of the cultivated turmeric varieties.

INTRA-SPECIES VARIATION IN NUCLEAR DNA CONTENT OF TURMERIC VARIETY OF NORTH EAST INDIA

5C.1 Introduction

Turmeric (*C. longa* L. Zingiberaceae), is widely cultivated and extremely marketable spice in Asian country, having distinctive chemical and physical properties. The plant has wide industrial applications, like manufacture of canned beverages, baked product, farm product, ice cream, yogurt, yellow cakes, fruit crush, biscuits, popcorn color, sweets, cake icings, cereals, sauces, gelatins, and curry powders etc. Turmeric is employed as an additive, preservative and coloring agent in Asian countries (Antunes and Araujo 2000; Cecílio-Filho et al 2000).In addition, turmeric has a wide range of medicinal activities, with recent findings that shows that curcumin, the yellow color pigment of turmeric, may be a powerful inhibitor, anti-parasitic, antispasmodic agent and anti-inflammatory compound, which can inhibit carcinogenesis (Araujo & Leon 2001; Ravindran 2007).Indian enjoys monopoly in turmeric production and export. Owing to its ever-increasing demand in food and pharmaceutical industries, there is an urgent need to increase the productivity of turmeric. However, attainment of increase in productivity, data concerning the crop's genetic diversity is crucial for breeding programs (Nass 2001). Moreover, McMurphy & Rayburn (1991) noted that the more intensive the breeding programme involved in the development of a cultivar, the lower the association between DNA variability and agronomic characteristics.

While at first this looks to be of very little consequence, it should be realized that nuclear DNA content variation in crop species (or their primitive relatives) is being lost because of the slender variety of nucleotides within the breeding pool. This nucleotypic variation may play a vital role within the adaptation of crop species to dynamical environmental conditions. It's been documented that the eukaryotic DNA will adapt to

numerous stresses by DNA amplification in each animal and plant cells. Sequence amplification is purported to be a conducive mechanism to the fluidity of the organism genome (Bachmann 1993). Additionally, if a breeding concept induced unwanted DNA content variation into a plant choice, this variability may well beharmful to the breeding programme. Such surprising DNA content variation has been seen throughout the assembly of F" hybrid maize (Rayburn et al 1993). The nuclear DNA content variation of 15.1 % has already been documented in Indian accessions of turmeric (Skornickova et al 2007). The cytophotometric estimation of nuclear DNA content of 17 varieties of *C. longa* ranged from 4.30 to 8.84 pg (Nayak et al 2006). In addition, flow-cytometric nuclear DNA amounts for16 taxa (including the above-mentioned species and one undetermined sample) from Bangladesh is given in the unpublished PhD thesis of Islam (2004). The genome size variation of 1.07 fold has been documented for *C. zedoaria* from the wild accessions of Bangladesh. In this current study, the nuclear DNA content variation in cultivated variety of turmeric has been studied.

5C.2 Materials and methods

5C.2.1 Plant materials

For details section 4A.2.1 can be referred.

5C.2.2 Standards for flow cytometric estimation of nuclear DNA content

Seeds of *O. sativa* 'IR36' (2C = 1.01 pg, Price and Johnston 1996), *S. lycopersicum* cv. Stupicke (2C = 1.96 pg, Dolezel et al 1992), were used as the standards for flow cytometric estimation of nuclear DNA content in the turmeric germplasm of NE India.

5C.2.3 Sample preparation and flow cytometric estimation

The details of the sample preparations and flow cytometric estimation were discussed in section 5A.2.3 and section 5A.2.8, respectively.

5C.3 Results and discussion

5C.3.1 Inter-varietal variation in nuclear DNA content

The nuclear DNA content of the 19 cultivated turmeric varieties against one internal standards (*O. sativa*) is shown in Figure 5C.1 and presented in tabular format (Table 5C.1). There were significant differences ($p < 0.05$) in nuclear DNA content. Eleven groupings of turmeric varieties with significant differences in nuclear DNA content were identified against *O. sativa* as an internal standard including four different ploidy levels (5x, 6x, 7x and 9x).

The DNA content of cultivated variety of turmeric2 & 6 were significantly higher than that of any other cultivated varieties (group j in Table 5C.1) (2C -value = 2.76-2.81 pg, *p*- value = 0.116). Considering 1-C value of turmeric to be 0.30 (Skornickova et al 2007), the ploidy level of the germplasm 2 and 6 is 9x. These nona-ploid varieties were followed by low-nonaploid groups of statistically similar nuclear DNA content [group i (5 &13): 2C-value = 2.60-2.63 pg, *p*-value = 0.961; group h (5 & 10):2C = 2.56-2.60, pg, *p*-value = 0.391; and g (3 & 10): 2C-value = 2.54-2.56 pg, *p*-value = 0.989] (Table 5C.1). So, the percentage of nonaploids in the studied turmeric cultivars is 30.0%. This is followed by the heptaploid group of solitary cultivated turmeric variety having 2C value of 2.20 pg found in Assam (Cultivated variety 1) providing 5 % coverage of the studied cultivated varieties. The next statistically similar and the largest groupings (58 % of the studied varieties) was of hexaploid varieties [group e (7, 8, 9, 16, 17 and 19): 2C-value = 1.82 - 1.87 pg, *p*-value = 0.182; group d (8, 9, 11, 12, 16, 17, 18 and 19) 2C-value = 1.80 - 1.86 pg, *p*-value = 0.116; group c (9,11,12,14, 16, 17, 18 and 19) 2C-value = 1.80 - 1.84 pg, *p*-value = 0.672)]. The next statistical solitary group was observed to be the turmeric variety 4 having 2C-value of 1.60 pg with low hexaploidy status. One variety from Meghalaya (variety no 15 with 2C= 1.36 pg) was found to be having pentaploidy status (5x).

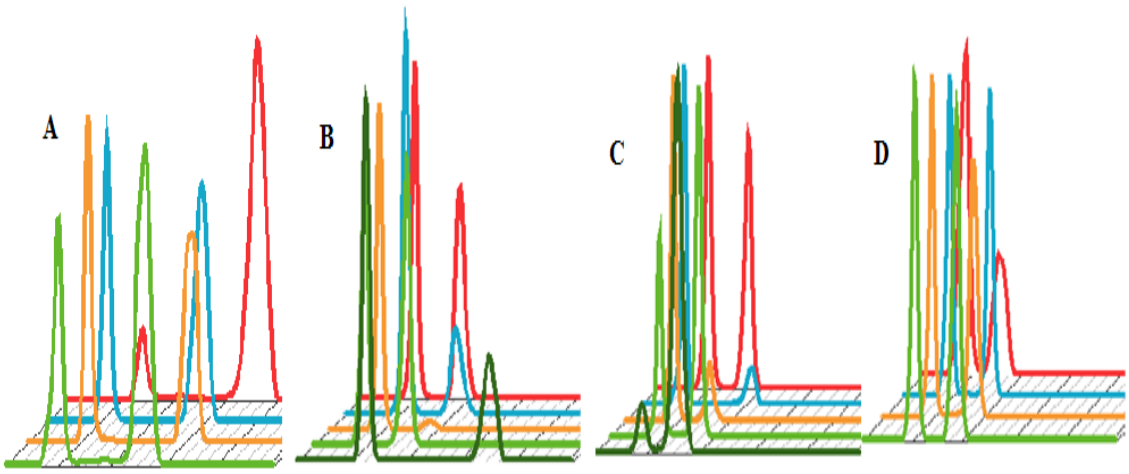

Fig 5C.1 Histogram of fluorescence intensity of the nuclear DNA content of 19 turmeric variety estimated by internal standardization with *O. sativa* 'IR36'. **A.** Turmeric variety of Assam (12951, 12953, 12955, 12950, 12956); **B.** Arunachal Pradesh (12952, 12940, 12949, 12947, 122931); **C.** Manipur (12957, 12978, 12989,12939, 12910); **D.** Meghalaya (12980, 12944, 12990, 12985)

Among the all studied turmeric varieties, the highest nuclear DNA content was found to be 2.83 pg and the lowest nuclear DNA content was found to be 1.35 pg with the fold variation of 2.10. The mean nuclear DNA content of turmeric was observed to be 2.08 pg. Positive correlation (r = 0.63) was observed between the average nuclear DNA content of each variety and Shannon's information index (Refer to Table 4A.3), implying genetic diversity increases with increase in nuclear DNA content. Significant variation of 4C DNA content was recorded at the intra-specific level with values ranging from 4.30 to 8.84 pg. The differential DNA content observed among 17 different cultivars of *C. longa* comprising same ($2n$ = 48) chromosome number could be attributed to the loss or addition of highly repetitive sequences in the genome (Nayak et al 2006). Skornickova et al (2007) showed the nonaploids (9x) with 15.1% intra-specific variation for their turmeric variety analysed from India. If polyploids are the reasons for the production of turmeric, the polyploids (9x is 60%) studied in the turmeric production were less compared to hexaploids. This is one of the predicted reasons of low productivity of the north eastern turmeric varieties.

India is responsible for around 90% of turmeric production worldwideand this species has been widely used in India since Vedic times. Perhaps the variation may have adaptive value, as previously documented in another crop, *Zea mays (*Rayburn & Auger 1990). The reasons for intra-specific genome size variation in *Curcuma* remain unknown. Aneuploidy or presence of B-chromosomes may lead to heterogeneity in nuclear DNA amount, but this explanation seems rather unlikely as only euploid numbers were revealed and more importantly, two accessions of *C. longa* with more than 9% genome size variation possessed the same number of chromosomes (Skornickova et al 2007). Plausibly, intra-specific variation may be related to a long-term cultivation and targeted selection of desirable genotypes in several *Curcuma* species (Skornickova et al 2007).

Table 5C.1 Variation in nuclear DNA content as estimated using flow cytometry

Population	Code	2C-DNA content (pg)	CV	Ploidy status	Minimum–Maximum (range) (2C)	Fold variation
Assam	1	2.21^{f}± 0.018	3.21	7x	1.58-2.74	1.73
	2	2.74^{j}± 0.002	2.81	9x		
	3	2.56^{g}± 0.010	2.83	9x		
	4	1.58^{b}± 0.032	2.47	6x		
Arunachal	5	$2.59^{h,i}$± 0.021	3.00	9x	1.84-2.83	1.73
	6	2.83^{j}± 0.021	2.86	9x		
	7	1.87^{e}± 0.012	3.73	6x		
	8	$1.87^{d,e}$± 0.009	3.29	6x		
	9	$1.84^{c,d,e}$± 0.011	3.29	6x		
Manipur	10	$2.56^{g,h}$±0.018	3.06	9x	1.35-2.63	1.95
	11	$1.83^{c,d}$± 0.021	2.76	6x		
	12	$1.79^{c,d}$±0.018	2.98	6x		
	13	2.63^{i}± 0.021	3.05	9x		
	14	1.79^{c}±0.018	3.14	6x		
Meghalaya	15	1.35^{a} ± 0.021	2.23	5x	1.83-1.85	1.01
	16	$1.86^{c,d,e}$±0.002	2.96	6x		
	17	$1.84^{c,d,e}$±0.018	3.39	6x		
	18	$1.83^{c,d}$± 0.012	4.02	6x		
	19	$1.83^{c,d,e}$±0.018	2.80	6x		
Mean		2.08 ± 0.43			1.35 – 2.83	2.10

The super-scripts of the same letters have statistically similar nuclear DNA content (p=0.05)

5C.3.2 Intra- varietal variation in nuclear DNA content

The highest nuclear DNA content of the turmeric variety (2) of Assam was found to be 2.74 pg (9x) and the lowest nuclear DNA content of the same (3) was found to be 1.58 (6x) pg with the fold variation of 1.36. Among the five varieties screened from Assam the 9x ploidy stature was observed for three cases and the rest varieties were 6x and 7x.

The highest nuclear DNA content of Arunachal varieties of turmeric (6) was found to be 2.83 pg (9x) and the lowest nuclear DNA content (9) of the same was found

to be 1.84 pg (6x) with the fold variation of 1.73 fold. Two nonaploids and three hexaploids varieties were observed among the studied species of the state.

The highest and the lowest nuclear DNA content observed for Manipur varieties was 2.63 pg and 1.35 pg, respectively. The fold variation was found to be 1.95. One nonaploid, three hexaploid varieties and one pentaploid variety was observed.

The fold variation of nuclear DNA content was low in Meghalaya varieties of turmeric. It is 1.01 fold. The maximum nuclear DNA content was observed to be 1.96 pg and the minimum nuclear DNA content was found to be 1.83 pg. Skornickova et al (2007) observed that for the genus *Curcuma*, most of the varieties are hexaploid in nature. This allows them to hypothesize that these hexaploids have played a key role in the evolution of polyploidy in Indian *Curcuma* spp. Nonaploid cytotypes probably originated by a fusion of reduced and unreduced gametes of hexaploids, either within or between species, giving rise to auto - or allopolyploids (Skornickova et al 2007).

Having the lowest fold variation of nuclear DNA content for Meghalaya variety could point to the temperature of the hilly states and over exploitation of the varieties for turmeric production. The fold variation in nuclear DNA content was found to be minimum for varieties occurring in Manipur.

5C.4 Conclusion

It was observed in current study that an overall 2.10 fold variation exist among the turmeric (*C. longa*) varieties. Turmeric varieties of state Meghalaya was less varying compared to the rest of the varieties. The ploidy status of the studied turmeric varieties varied from 5x to 9x type. This is the first study on the nuclear DNA content of turmeric varieties of NE India. The study will help in assessing the ploidy status of the turmeric varieties. The correlation study of the productivity and the ploidy status shall further help in devising the strategy whether ploidy screening could be potential marker for crop productivity.

CHAPTER 5D

NUCLEAR DNA CONTENT ESTIMATION OF ZINGIBEROIDEAE

.

This chapter describes the nuclear DNA content estimation of Zingiberoideae species by internal standardisation.

NUCLEAR DNA CONTENT ESTIMATION OF ZINGIBEROIDEAE

5D.1 Introduction

Flow cytometry has been used with a number of different plant species for determining cell and nuclear DNA content (Galbraith 1990), analyzing cell cycles (Bergounioux & Brown 1990), determining chromosome karyotypes, sorting cells and chromosomes, and characterizing other cellular parameters (Rayburn et al 1989, 1992; Fuchs & Pauls 1992; Liu et al 1997). DNA C-value remains a key character in biology and biodiversity. Genome size has many important practical implications at many different levels. For example, species with large DNA amounts (i.e. 1C greater than 20 pg) can be problematic when studying genome diversity using the standard AFLP™ technique [designed for genomes of 500± 6000 Mbp (approx. 0.5±6 pg); Perkin-Elmer, 1996] with three selective bases on each primer, and it may be necessary to increase the number of selective bases or to change the restriction enzymes. Nuclear DNA content remains a basic parameter in determining a species is suitable for genetic diversity studies by AFLP markers. Moreover, possession of a very small DNA content has been a major factor in determining which taxa were chosen as the first candidates for genome sequencing, and which chromosome(s) in the karyotypes of various organisms were sequenced first. *Arabidopsis thaliana* was the first plant chosen for genome sequencing, partly because it had one of the smallest C-values known for an angiosperm (NSF, 1990; Anderson 1991). A grass in the genus *Brachypodium* (e.g. diploid B. distachyon1C = 0.25±0.3 pg) was proposed as a first monocot for genome sequencing on similar grounds (Bablak et al 1995; Catalan et al 1995), but rice (*Oryza sativa*, 1C . approx. 0.5 pg) was chosen because it has the smallest C-value among the world's major cereal crops (Sasaki 1998; Somerville and Somerville 1999).

Despite its usefulness in understanding plant evolution and diversification, genome size variation is not well documented in Indian subcontinent and estimates

for only a few species have been published (Skornickova et al 2007). Bharathan et al (1994) determined by flow cytometry determined 1C = 1.30 pg in *C. zanthorrhiza* and Das et al (1999) used cytophotometry to study genome size in *C. amada* (4C = 3.120 pg), *C.caesia* (4C = 4.234 pg) and *C. longa* (4C = 5.100–5.263 pg). *Curcuma longa* was also investigated by Nayak et al (2006), who observed 4C-values ranging from 4.30 to 8.84 pg in 17 varieties. In addition, flow-cytometric nuclear DNA amounts for 16 taxa (including the above-mentioned species and one undetermined sample) from Bangladesh are given in the unpublished PhD thesis of Islam (2004). The 2C-DNA content of the sub family Zingiberoideae (Zingiberaceae) of NE India is to be done.

5D.2 Materials and methods

5D.2.1 Plant Materials

A total of 26 species were taken into account (Table 5A.1). For details, section 5A.2.1 can be referred.

5D.2.2 Standards for flow cytometric estimation of nuclear DNA content

For details, section 5A.2.2 can be referred.

5D.2.3 Isolation and staining of nuclei and flow cytometric analyses

For details section 5A.2.3 and section 5A.2.8 can be referred.

5D.3 Results and discussion

5D.3.1 Nuclear DNA content for Zingiberoideae species by internal standards

Clearly defined histograms were obtained for the four standard species having CV of < 5.00 % (Fig 5D.1). However, the nuclear DNA content varied among the replicate measurements among different standards. So it was necessary to execute a statistical regression analysis to find the nuclear DNA content of each of the Zingiberoideae species. A straight-line relationship was observed between the DNA amounts of the four standards. The coefficient of determination (r^2) was 1.000 and the ratio of the PI

fluorescence intensities of the standards to Zingiberaceae plants (p1/p2) was defined by the formula:

$$\text{DNA amount (pg)} = a\frac{p1}{p2} \pm b$$

Where, a is the integer value and b is the y axis intercept. Similarly the regression equation for the other species was also obtained. By putting the x = 1 in the regression equation, we obtained the nuclear DNA content for *H. coronarium*to be 4.73 pg. The 2C DNA amount of each Zingiberaceae was estimated as the mean of four replicates and the standard deviation was calculated using the formula:

$$s.d = \sqrt{s.d_S^2 + s.d_R^2}^2$$

The regression equation along with the coefficient of determination (r^2) has been shown for *C. aromatica* (Fig 5D.2). The other regression equations, coefficient of determination, nuclear DNA content of each of the 26 species with four different standards has been given shown in Table5D.1. Based on the nuclear DNA content data, the phylogenetic tree was constructed (Fig 5D.3**)**.

The majority of Indian *Curcuma* is reported to possess the basic chromosome number x = 7, published count corresponds to 6x, 9x, 11x, 12x and 15x ploidy levels. *C. zedoaria* locally named as `keturi´, the published chromosome number and nuclear DNA content is 2n =63, 64, 66 and 2C = 3.285 pg (Islam et al 2004). In our study the 2n chromosome number and nuclear DNA content of *C. zedoaria* was found to be 2n = 63 and the 2.78 pg based on four different internal standards. The ploidy level of *C. zedoaria* was experimentally found to be nonaploid (9x) depicting 1Cx value of 0.31 pg. Our estimated value of nuclear DNA content for *C. zedoaria* of 2.78 pg was is close agreement with the value estimated by Skornicova et al 2007. *C. amada* locally named as `amada´, is considered to be hexaploid (6x), having chromosome count of 42. Its genome size is reported in the range of 1.810 ± 0.002 to 1.882 ± 0.002 with 3.6 % intra-species variation (Skornicova et al 2007, based on Eastern and North eastern parts of India) whereas in our case it is reported to be 1.858 ± 0.006 (based on North Eastern parts of India) which is closely following the published reports. The taxa is a natural hexaploid and we can predict the C_x value to be0.31 pg.

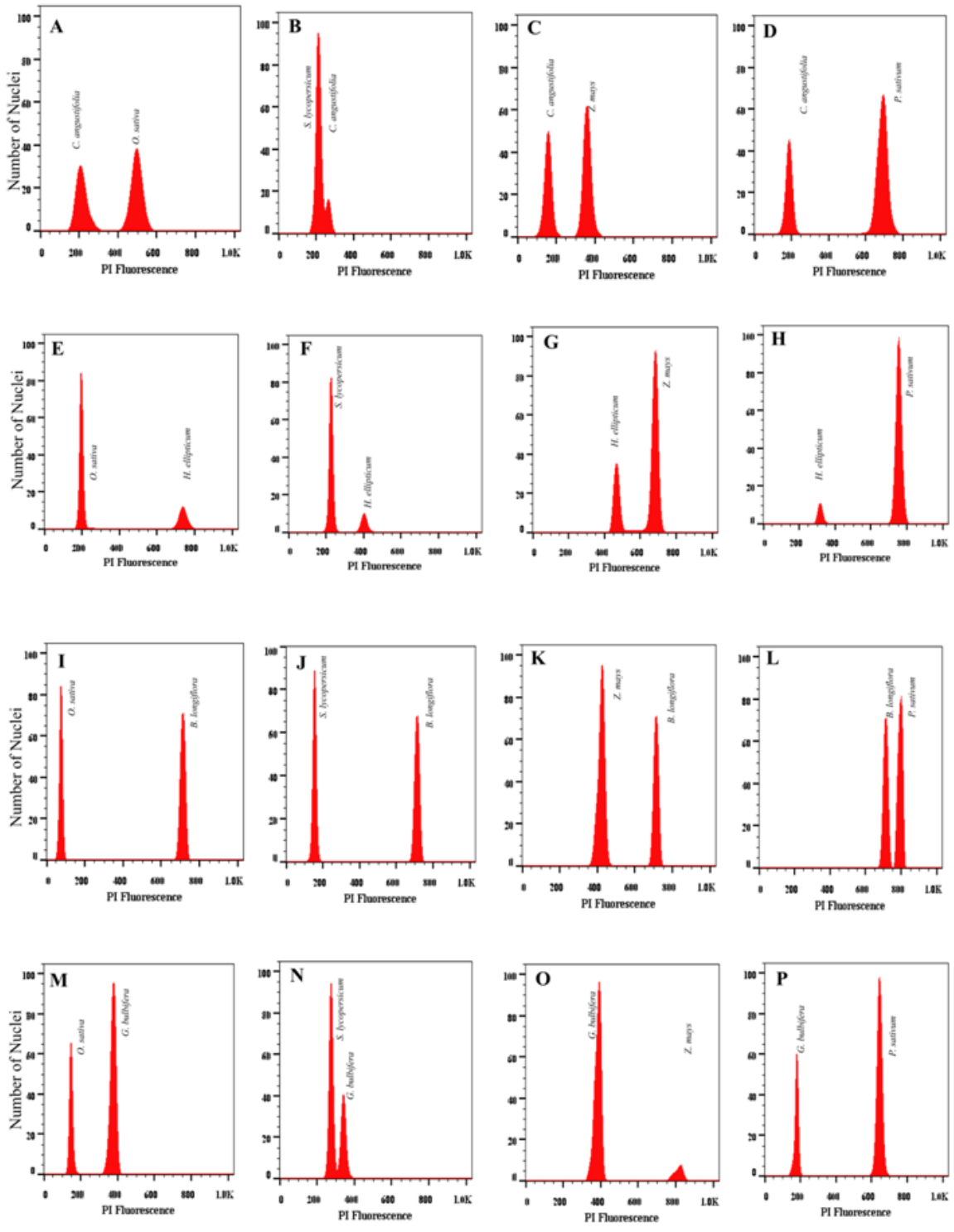

Fig 5D.1 Histogram of fluorescent intensity of four Zingiberaceae species using four different internal standards. The species in the panel are: *C. angustifolia*, *H. ellipticum*, *B. rotunda and G. bulbifera.* The following reference standards were used *Oryza sativa* 'IR 36' (2C = 1.01 pg DNA) (a, e, i and m), *Solanum lycopersicum*'Stupicke´' (2C = 1.96 pg DNA) (b, f, j and n), *Zea mays* 'CE-777' (2C = 1.96 pg DNA) (c, g, k, o) and *Pisum sativum* cv. 'Citrad' (2C = 1.96 pg DNA) (d, h, k, p).

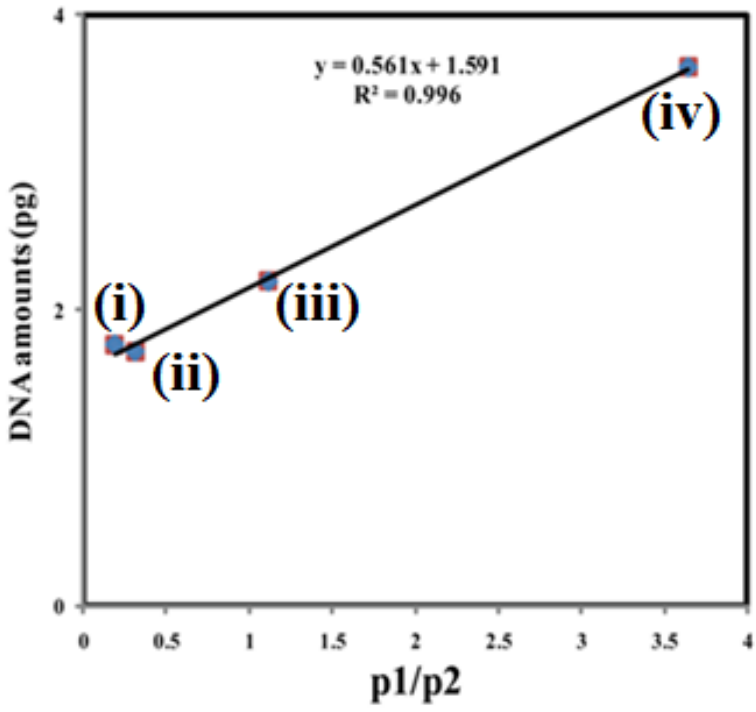

Fig 5D.2 Regression analysis of four flow cytometric standards. Nuclear DNA content of four internal standards used in relation to the ratio of median fluorescence intensities of PI-stained nuclei of the standards to *C. aromatica (*p1/p2). (i) *P. sativum,* (ii) *Z. mays,* (iii) *S. lycopersicum* (iv) *O. sativa.*

Table 5D.1 DNA content of 26 Zingiberaceae plants determined by flow cytometric analysis of propidium-iodide-stained nuclei using internal reference standards.

Serial No	Genus	Internal Standard used	DNA content obtained (2C)by each standard (pg) ± s.d	Coefficient of variation (CV)	Regression equation and coefficient of determination (r^2)	Nuclear DNA content (pg/2C)			
						This study		Previous study	
						(pg) /2C	Mbp / 1C	(pg) /2C	reference
1	*Curcuma zedoaria*	*O. sativa*	2.84 ± 0.01	4.07	y = 0.012x + 2.768	2.780±0.020	1359	3.15-3.37	Islam et al 2007
		S. lycopersicum	2.70 ± 0.02	4.70	r^2= 0.967				
		Z. mays	2.67 ± 0.04	4.75					
		P. sativum	2.91 ± 0.01	3.60					
2	*Curcuma amada*	*O. sativa*	1.81 ± 0.02	2.84	y = 0.017x + 1.841	1.858±0.006	909	1.86	Skornickova et al 2007
		S. lycopersicum	1.83 ± 0.02	4.90	r^2= 0.958				
		Z. mays	1.84 ± 0.01	3.52					
		P. sativum	1.82 ± 0.03	3.86					
3	*Curcuma angustifolia*	*O. sativa*	2.49 ±0.09	4.27	y = 0.044x + 2.395	2.439±0.029	11.93	2.15	Skornickova et al 2007
		S. lycopersicum	2.48 ±0.04	4.53	r^2= 0.998				
		Z. mays	2.36 ±0.07	4.79					
		P. sativum	2.45 ±0.03	3.71					
4	*Curcuma domestica*	*O. sativa*	2.57 ± 0.02	3.0	y = 0.005x + 2.518	2.523±0.006	1234	-	-
		S. lycopersicum	2.54 ± 0.01	4.87	r^2= 0.975				
		Z. mays	2.47 ± 0.05	3.57					
		P. sativum	2.57 ± 0.04	3.33					
5	*Curcuma longa*	*O. sativa*	2.57 ± 0.03	2.03	y = 0.005x + 2.518	2.523±0.020	1234	2.71	Skornickova et al 2007
		S. lycopersicum	2.53 ± 0.01	2.47	r^2= 0.989				
		Z. mays	2.47 ± 0.02	3.44					
		P. sativum	2.56 ± 0.04	2.98					
6	*Curcuma leucorrhiza*	*O. sativa*	1.66 ± 0.02	2.64	y = - 0.044x +	1.681±0.006	822	2.70	Skornickova et

		S. lycopersicum	1.56 ± 0.01	3.93	1.725 r^2= 0.977				al 2007
		Z. mays	1.75 ± 0.05	3.04					
		P. sativum	1.76 ± 0.02	3.69					
7	*Curcuma* caesia	*O. sativa*	1.80 ± 0.02	3.87	y = - 0.149x +	2.092±0.029	1023	-	-
		S. lycopersicum	1.96 ± 0.01	4.23	2.241 r^2= 0.988				
		Z. mays	1.84 ± 0.03	2.15					
		P. sativum	1.86 ± 0.04	4.5					
8	*Curcuma* species2	*O. sativa*	2.73 ± 0.02	3.22	y = 0.013 x +	2.676±0.020	1308	-	-
		S. lycopersicum	2.73± 0.01	3.22	2.663 r^2= 0.990				
		Z. mays	2.58 ± 0.03	3.86					
		P. sativum	2.66 ± 0.06	2.35					
9	*Curcuma* species3	*O. sativa*	2.78 ± 0.01	3.87	y = 0.149 x +	2.390±0.006	1169	-	-
		S. lycopersicum	2.76 ± 0.05	4.29	2.241 r^2= 0.987				
		Z. mays	2.73 ±0.02	1.93					
		P. sativum	2.65 ± 0.04	2.35					
10	*Curcuma aromatica*	*O. sativa*	3.64 ± 0.02	3.49		2.890±0.029	1052	2.83	Skornickova et al 2007
		S. lycopersicum	2.19 ± 0.03	2.92	y = 0.561 x + 2.320				
		Z. mays	1.72 ± 0.04	2.85	r^2= 0.996				
		P. sativum	1.77± 0.03	4.88					
11	*Hedychium coronarium*	*O. sativa*	2.07± 0.02	4.73	y = - 0.156 x +	1.967±0.006	962	-	-
		S. lycopersicum	1.83 ± 0.07	4.88	2.123 r^2= 0.992				
		Z. mays	0.97 ± 0.04	3.89					
		P. sativum	2.08 ± 0.05	4.58					
12	*Hedychium chrysoleucum*	*O. sativa*	2.19 ± 0.03	4.11	y = - 0.044x +	2.151±0.020	1052	-	-
		S. lycopersicum	0.98 ± 0.02	3.14	2.195 r^2= 0.956				
		Z. mays	2.12 ± 0.01	3.67					
		P. sativum	2.26 ± 0.06	4.70					
13	*Hedychiumspicatum*	*O. sativa*	2.90 ± 0.03	4.28	y = - 0.140 x +	3.512±0.006	1718	2.94	Nag et al 2011
		S.	3.90 ± 0.01	4.10	3.652				

		lycopersicum			r^2 = 0.999				
		Z. mays	2.50 ±0.03	4.63					
		P. sativum	4.48 ± 0.03	3.90					
14	*Hedychiumgardnerianum*	*O. sativa*	4.44 ± 0.02	4.81	y = 0.020 x + 4.280	4.300±0.020	2103	-	-
		S. lycopersicum	4.27 ± 0.04	2.66	r^2= 0.983				
		Z. mays	4.17 ± 0.02	2.44					
		P. sativum	4.41 ± 0.05	1.99					
15	*Hedychiumellipticum*	*O. sativa*	3.76 ± 0.03	2.83		3.702±0.006	1810	-	-
		S. lycopersicum	3.46 ± 0.05	2.99	y = - 0.011 x + 3.713				
		Z. mays	3.72 ± 0.02	2.12	r^2= 0.966				
		P. sativum	3.83 ± 0.01	2.15					
16	*Kaempferia galanga*	*O. sativa*	8.11± 0.02	1.66	y = 0.005x + 7.961	7.966±0.020	3895	9.73	Chandrmai et al 2003
		S. lycopersicum	7.71 ± 0.03	4.97	r^2= 0.997				
		Z. mays	8.15 ± 0.01	3.92					
		P. sativum	-	-					
17	*Kaempferia angustifolia*	*O. sativa*	5.11 ± 0.02	4.73	y = 0.077x+ 4.646	4.723±0.006	2310	4.67	Chandrmai et al 2003
		S. lycopersicum	4.65 ± 0.04	3.45	r^2= 0.900				
		Z. mays	4.83 ± 0.01	2.03					
		P. sativum	-	-					
18	*Kaempferia pulchra*Ridl.	*O. sativa*	6.98 ± 0.04	4.80	y = 0.063x+ 6.436	6.499±0.029	3178	4.13	Chandrmai et al 2003
		S. lycopersicum	6.36 ± 0.02	3.08	r^2= 0.900				
		Z. mays	6.70 ± 0.01	2.50					
		P. sativum	-	-					
19	*Kaempferia pulchra*Ridl. 1	*O. sativa*	3.42± 0.02	2.20	y = -0.019x + 3.508	3.489±0.035	1706	3.49	Chandrmai et al 2003
		S. lycopersicum	3.55± 0.04	2.67	r^2= 0.975				
		Z. mays	3.20± 0.02	4.10					
		P. sativum	3.74± 0.04	2.12					
20	*Kaempferia rotunda*	*O. sativa*	3.47± 0.02	4.76	y = 0.028x +3.514	3.542±0.020	1732	4.43	Chandrmai et al 2003
		S. lycopersicum	3.86± 0.03	4.95	r^2= 0.990				

		Z. mays	3.38± 0.01	4.20					
		P. sativum		-					
	Globba bulbiflora	*O. sativa*	2.56 ± 0.01	3.85	y = -0.009x +	2.531±0.006	1238	-	-
21		*S. lycopersicum*	2.42 ± 0.02	4.79	2.540 r^2= 0.954				
		Z. mays	2.59 ± 0.05	3.83					
		P. sativum	2.55 ± 0.03	4.09					
	Boesenbergia rotunda	*O. sativa*	9.29 ± 0.04	3.86	y = -0.747x +	6.441±0.006	3150	-	-
22		*S. lycopersicum*	8.93 ± 0.03	3.74	7.188 r^2= 0.999				
		Z. mays	9.02 ± 0.05	3.46					
		P. sativum	7.96 ± 0.03	3.02					
	Zingiber officinale	*O. sativa*	3.79 ± 0.04	4.06	y = 0.063x +	3.608±0.029	1764	12.05	Rai et al 1997
23		*S. lycopersicum*	3.65 ± 0.02	4.20	3.545 r^2= 0.987				
		Z. mays	3.59 ± 0.04	4.95					
		P. sativum	-	-					
	Zingiber moran	*O. sativa*	3.71 ± 0.03	4.87	y = 0.025x +	3.689±0.020	1804	-	-
24		*S. lycopersicum*	3.83 ± 0.02	4.63	3.664 r^2= 0.966				
		Z. mays	3.61 ± 0.01	1.97					
		P. sativum	-	-					
	Costus speciosus	*O. sativa*	3.74± 0.02	-	y = -0.117x +	3.895	1904	-	-
25		*S. lycopersicum*	-	-	4.012 r^2= 0.938	±0.006			
		Z. mays	3.55 ± 0.02	4.74					
		P. sativum	4.58 ± 0.05	4.86					
26	*Alpinia nigra*	*O. sativa*	4.44± 0.01	-	y = - 0.156 x	4.587±0.020	2243	-	-
		S. lycopersicum	4.39 ± 0.04	2.38	+4.743 r^2= 0.956				
		Z. mays	4.64 ±0.06	2.50					
		P. sativum	4.64 ±0.03	2.89					

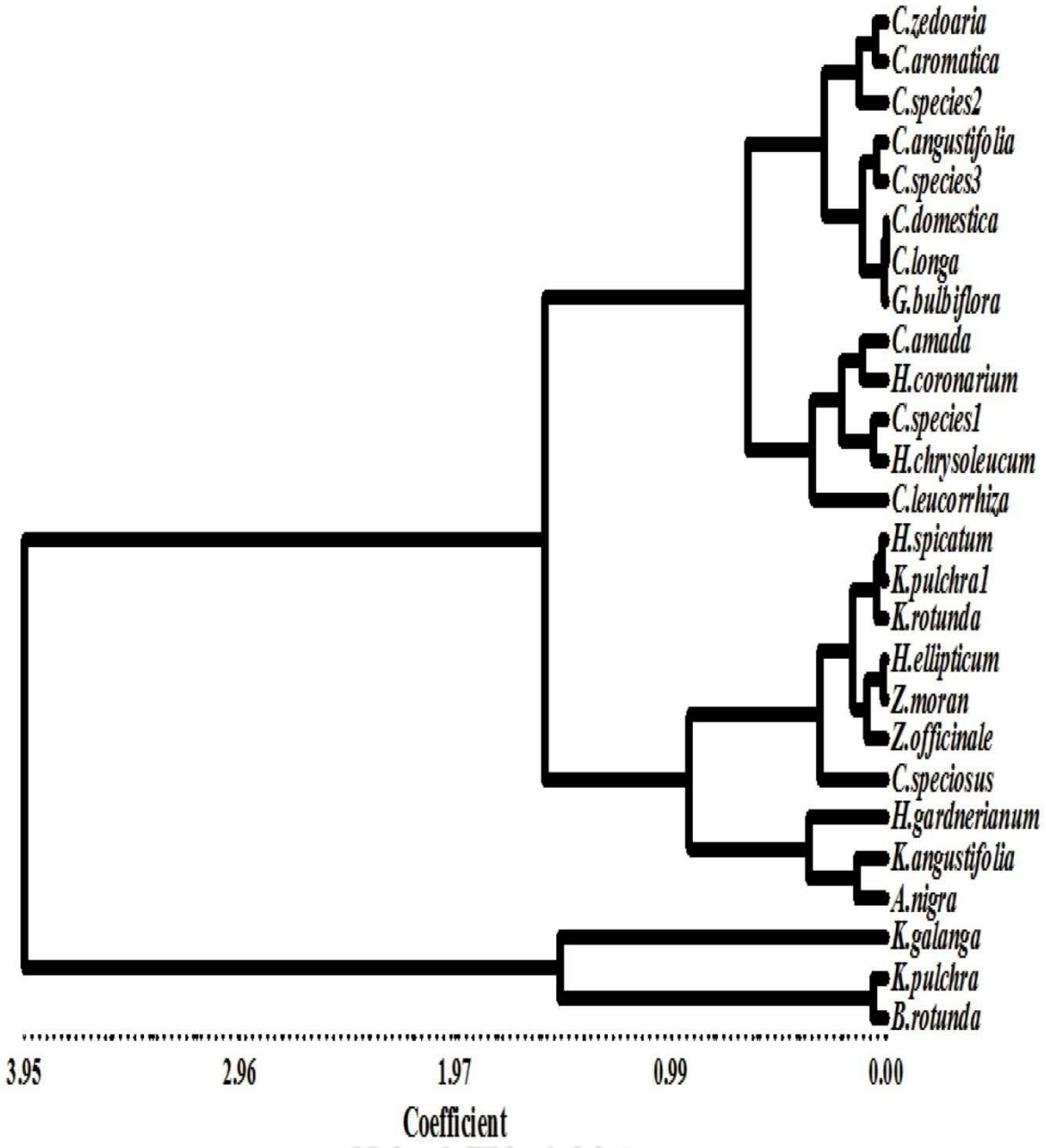

Fig 5D.3 Evolutionary tree constructed based on SHAN module of NTYSYS pc was chosen for the analysis, using the Manhattan distance as a dissimilarity coefficient.

Asian *C. angustifoila* (Gorusat haladhi) is reported to be hexaploid having 2n = 42 chromosomes with 2C value of 3.289 pg (Islam 2004, based on population from Bangladesh), 2.148 ± 0.003 (Skornicova et al 2007, population of Northern and Eastern parts of India), whereas our estimate is in the middle of the two reported 2C value (2.439 ± 0.003 pg) based on the taxa of NE parts of India. The deviation from the estimated value of nuclear DNA content of *C. angustifolia,* we believes due to the intra species variation because of geographical isolation. *C. longa* locally named as `Haladhi´, is nonaploid (9x) bearing chromosome number of 63 (Das et al 1999), 2C-value in the range of 2.58 to 2.82 pg with 15.1% intra species variation (Skornicova et al 2007, based on populations of all over India). Our estimated 2C- value of *C. longa,* 2.523 ± 0.020 pg (population belonging to Barpeta district of Assam) was found to be close to the reported 2C value originated from Assam and West Bengal.*C. domestica* locally named as `Haldi´ having high curcumin content is found to possess the same nuclear DNA content as that of *C. longa.* So high curcumin content is not causing significant variation in the chromatin organization since PI uptake was found to be equivalent. *C. leucorrhiza* is also reported to be nonaploid taxa having 2C-value of the order of 2.688 pg to 2.692 pg with the estimated mean value of 2.70 pg (Skornicova et al 2007, based on Eastern part of India). Our experiment revealed 1.68 ± 0.006 pg (based on the *C. leucorrhiza* collections) of NE India. The acquired result signifies it is two third of the reported estimates. Further work needs to be carried out on the chromosome number and the ploidy determination to confirm its hexaploidy (6x) stature. *C. aromatica* locally known as `Bonhaladi´ is also a nonaploid taxa with the reported chromosome number and 2C-value of 63 (Ramachandran 1961) and 2.815-2.869 with 1.7% intra species variation (Skornicova et al 2007, based on populations of North eastern parts of India), 42 (Raghavan &Venkat 1943) and 1.86 pg with 3.5% intra species variation (Skornicova et al 2007), based on populations of southern parts of India and Srilanka. Our estimated 2C-value of *C. aromatica* was 2.89 ± 0.029 pg based on the population of the North-eastern parts of India. It can be predicted that the experimental taxa of *C. aromatica* was nonaploid since the experimental 2C-value was found similar to the nonaploid variety. Among the unidentified species of *Curcuma* from the Ultapani reserve forest of Assam,

nuclear DNA content was found to vary from 2.092 ± 0.029 to2.676 ± 0.020, indicating that the species are from same location with 1.28 fold variation in nuclear DNA content though there were morphological differences. In the genera *Curcuma*, the highest genome size was experimentally found out to be 2.89pg for *C. aromatica*. The lowest genome size was found out to be 1.68 pg for *C. leucorrhiza* representing 1.72 variations within the 10 studied *Curcuma* species. The fold variation found in the current study (1.72 fold) was found to be lower compared to earlier reported one which is 2.87 fold (Skornicova et al 2007).

Exhaustive literature survey on the published reports of the genera *Hedychium* (NE India consisting of 24 species out of 65 worldwide) resulted in the solitary species *H. spicatum* having 2C-value and chromosome number of 2.94 pg (population belonging to Western Himalaya) (Nag et al 2011) and 34 (Joseph 2010). In this work we have shown the 2C value of *H. spicatum* to be 3.512 ± 0.006 pg (based on population of NE India) and chromosome number of 2n = 34 (diploid). The previous report was basically a study based on 5 different species and based on only one standard (*Zea mays*) where two fold variations persisted between sample and standard species. Our experimental finding of 2C-value of 1.967 ± 0.006for *H. coronarium* (populations from NE India) was the first report in of its kind. Experimentally we have found the chromosome number of *H. coronarium* was to be 2n = 34 which matches with the previous count of 2n = 34 and 51 (Ramachandran 1968) suggesting that our estimate is of natural diploid. The chromosome number and 2C-value of *H. chrysoleucum* (populations from NE India) was found to be 34 and 2.151 ± 0.020 pg suggesting the diploid nature in this study. *H. gardnerianum* is naturally a diploid species with chromosome number 2n = 34 and 2C-value of 4.300 ± 0.020 pg (based on the populations of NE India). *H. ellipticum* is found to be the diploid species with chromosome number and 2C-value of 2n= 34 and 3.702 ± 0.006 pg, respectively (based on the populations of NE India). In genus *Hedychium***,** the highest genome size was experimentally found to be 4.40 pg for *H. gardnerianum* and the lowest genome size was experimentally found out to be 2.06 pg for *H. coronarium*. The fold variation was reported to be 2.14 within the genus *Hedychium.*

The chromosome number and 2C-value of *K. galanga* were reported to be 2n = 44 and 9.73 pg (Chandrmai et al 2012), 2n = 54 (Joseph 2010). Whereas our experimental finding of chromosome number and 2C-value shows 2n = 55 and 7.966 ± 0.020 pg based on three standards. The variation in the nuclear DNA content is due to the intra species variation since the 2n = 9.73 were reported on the Indonesian accessions. Moreover only one standard *S. lycopersicum* (2C = 1.96 pg) was used as standard (Chandrmai et al 2012). *K. galanga* 2C value of 7.966 ± 0.020 is based on three standards (*O. sativa*, *S. lycopersicum* and *Z. mays*). Since *Kaempferia* has a basic number of 11 (Mahanty 1970), we can say *K. galanga* can be pentaploid having 1Cx value of 1.59 pg. *K. angustifolia* chromosome number and 2C value were reported to be 2n = 33 and 4.67 pg (Chandrmai et al 2012). Whereas our report on *K. angustifolia* estimated 2n = 22 and 4.723 ± 0.006 pg. It can therefore be said that accession is basically diploid with the 1Cx value equals 1C value i.e 2.36 pg. Chromosome number and 2C value of *K. pulchra* is reported to be 2n = 33 and 4.13 pg (Chandrmai et al 2012). For *K. pulchra*, we had two accessions. For the first accession, the genome size of 2C = 6.499 pg whereas for the second accession bearing purple flower, chromosome number and nuclear DNA content were experimentally found out to be 2n = 22 and 3.489 ± 0.035 pg. It would be interesting to know the chromosome number of *K. pulchra* first accession. Considering the second accession of *K. pulchra* as diploid, 1Cx value calculated as 1.75 pg. The chromosome number and 2C value of *K. rotunda* is reported to be 2n = 22 and 4.43 pg. Our estimate of nuclear DNA content of *K. rotunda* is 3.542 ± 0.020 pg.

From the genus *Globba*, one species was taken into consideration. The established report on the chromosome number suggest the diploid status of *Globba*2n = 32 (Eksomtramage et al 2002). There was no report on the nuclear DNA content of *G. bulbifera*and our estimate of *G. bulbifera* is 2.531 ± 0.006 pg. The chromosome number of *Boesenbergia rotunda* is reported as 2n = 20 (Eksomtramageet al 2002). Our estimated value of 2C = 6.441± 0.006 pg is the first report on *B. rotunda* to the best of our knowledge. Chromosome number and nuclear DNA content of *Z. officinale* (locally named as `ada´) is reported to have 2n = 22 and 2C = 12.05 (Rai et al 1997). Whereas we estimated 2C value to be 3.608 ± 0.029 pg. The huge variation in the nuclear DNA

content probably due to the technique used. *Z. moran*, a wild relative of *Z. officinale* possess chromosome number of 2n = 22 (Das et al 2013) estimated by our group earlier. In this study we report the nuclear DNA content of *Z. moran* to be 3.689 ± 0.029 pg. Chromosome number of *C. speciosus* was reported in the earlier studies as 2n = 36. Our estimated value of nuclear DNA content of *C. speciosus* as 3.895 ± 0.006 pg. This is also the first report on *C. speciosus.* The chromosome number of *A. nigra* is reported to be2n = 48 (Rangsiruji et al 2000). Our result suggests the 2C value of *A. nigra* is 4.587 ± 0.020. To the best of our knowledge this is the first report of nuclear DNA content of *A. nigra* as well. The nuclear DNA content of *A. speciosa* was reported to be 5.50 pg (Bharathan et al 1994).

We can class the Zingiberaceae into three groups, those that show little variation, those that show a moderate range of variation in genome size, and those that show wide range of variation in genome size. Most of the taxa (13/26) are of the first group (Class I), and may be characterized as having either small genomes in *Curcuma, Hedychium* and *Globba.* The second group (Class II) consisted of those species with moderate range of genome size in *Zingiber*, *Kaempferia*, *Costus* and *Hedychium*. The third group (Class III) is consisting of *Kaempferia* and *Boesenbergia* have the highest genome size. The minimum nuclear DNA content for *C. leucorrhiza* was reported to be 1.681 ± 0.006 pg. The maximum value of nuclear DNA content was reported for *K. galanga* was 7.966 ± 0.020 pg. The fold variation reported is 4.74 fold in this study.

5D.4 Conclusion

In this section the variation in nuclear DNA content was studied. The inter species variation in nuclear DNA content was found to be higher in *Kaempferia* followed by *Hedychium* and *Curcuma.* The fold variation was found to be 4.74 fold across the three genera.

CHAPTER 5E

DYNAMICS OF CHROMOSOME NUMBER AND NUCLEAR DNA CONTENT

This chapter deals with the correlation analysis of the chromosome number with nuclear DNA content variation

DYNAMICS OF CHROMOSOME NUMBER AND NUCLEAR DNA CONTENT

5E.1 Introduction

Genome size in flowering plants is a significant predictor of seed mass, flowering time, and habitat. Genome size varies widely, in flowering plants, providing ample variance for testing adaptive hypothesis. While large genomes have traditionally been assumed to be maladaptive, recent phylogenetic studies demonstrate both increase and decrease in flowering plant genome size. Recent studies also show weak support for the negative correlation between effective population size and genome size that would be expected under a scenario in which genome size inflation is enabled by drift in small populations. Mobile DNA elements are believed to be an essential source of genome size variation and genetic and phenotypic plasticity in eukaryotes. Understanding of the mobile DNA element makes its way from whole genome sequencing of the model organisms. Although the dynamics of mobile DNA element among the related taxa of natural populations and the role of mobile element at the species or supra-species level where evolution of chromosome number and nuclear DNA content are modulated, concert with polyploidy and chromosomal arrangement, remains poorly understood topic in any plant system. It has been proposed that increase in nuclear DNA content is contributed by the mobile DNA element proliferation and polyploidy- and of nuclear DNA content decrease is caused due to unequal recombination and indirect selection against nuclear DNA content (Chung et al 2012). An appropriate model group for evaluating the evolutionary dynamics of retro transposons on the micro-evolutionary scale should exhibit reasonable monoploid genome size variation within a particular taxon (section, subgenus, genus or family).

Nuclear DNA content is often used as a proxy for ploidy in comparative studies of chromosome variation (Ceccarelli et al 1995; Suda & Travnicek 2006; Suda et al 2007), based on the assumption that nuclear DNA content will vary with chromosome number atfine phylogenetic scales when chromosome number increases aredue to whole- or partial-

genome duplications. In organisms in which chromosome evolution is dominated by fission, fusion, and translocations, rearrangements that do not necessarily entail losses or gains of DNA, it is less clear what relationship should be expected between chromosome number and genome size, if any.

Among angiosperm speciesin which both the ploidy level (and chromosome number) and genome size are known (Bennett & Leitch 2012), one of the largest contrasts is reported in the genus *Eleocharis* (Cyperaceae).*E. acicularis* (2n = 20; 2C = 0.50 pg) and *E. palustris* (2n = 16; 2C = 11.05 pg) differ almost 22-fold in somatic DNA quantity at approximately the same ploidy level. This contrast is noticeable when compared with the majority of species in the family Cyperaceae, which exhibit smaller genomes. Extensive work on the chromosome number and the nuclear DNA content variation has been reported (Skornickova et al 2007). But surprisingly there is no report which shows the relationship of chromosome number and nuclear DNA content in Zingiberoideae. In this study, we investigate in a phylogenetic context the correlation between chromosome number and genome size for 9 species of Zingiberoideae.

5E. 2 Materials and methods

5E.2.1 Plant samples and method of analysis

In this study the plants for which chromosome numbers determined (9 species) already (section 3.3.6.5), were only taken into consideration to understand the behaviour of the differences in the chromosome number changes with nuclear DNA content. It includes three species of *Curcuma*, three species of *Hedychium* and three species of *Kaempferia* (Table 5E.1).

Table 5E.1 Species under consideration with their chromosome number and nuclear DNA amounts estimated in this thesis purview

S. no	Species	Chromosome number (2n)	Nuclear DNA content (2C)	Nuclear DNA content (1C)	2C (Mbp)/ 2n
1	*C. caesia*	21	2.09	1023	97.43
2	*C. longa*	42	2.523	1234	58.76
3	*C. zedoaria*	63	2.78	1359	43.14
4	*K. angustifolia*	22	4.723	2310	210
5	*K. pulchra*	22	3.489	1706	155.10
6	*K. galanga*	55	7.966	7.966	141.64
7	*H. chrysoleucum*	34	2.151	1052	61.88
8	*H. gardnerianum*	34	4.30	2103	123.71
9	*H. coronarium*	34	1.967	962	56.59

5E.2.2 Cytogenetics and nuclear DNA content

For details of cytogenetics analysis and nuclear DNA content estimation, section 3.3.6 and section 5A.2 can be referred.

5E.2.3 Data analysis

The nuclear DNA content experimentally determined in this thesis was only taken into consideration. When finding the correlation between the nuclear DNA content (2C) and chromosome number (2n), Pearson's correlation coefficient was performed in Sigma plot 11.0. The regression analysis (r^2) of the chromosome number and nuclear DNA content was carried out in Microsoft Excel 2007. Rescaling the datain this way has the effect of expressing chromosome number and genome sizein common units but has no effect on r^2or its significance. If chromosome number changes were due to unbiased deletion or duplication of chromosomes, the slope of this regression would approach 1.0 with increasing data. If chromosome number changes are due to fission and fusion with no change in nuclear DNA content, the regression slope will approach zero. This test assumes

that any chromosome deletions or duplications are unbiased with respect to the DNA content of each chromosome duplicated or deleted an assumption that is probably justified given the uniformity of chromosome sizes in Zingiberoideae.

5E.3 Results and discussion

Nuclear DNA content (2C) varied from 2.52 pg to 2.78 pg and chromosome number (2n) varied from 21 to 63 in the genus *Curcuma*. In genus *Kaempferia*, the variation of nuclear DNA content and chromosome number was 3.48 pg to 7.96 pg and 22 to 55, respectively. In the genus *Hedychium*, the variation of nuclear DNA content was in the range of 1.96 to 4.30 pg with no change in chromosome number (2n = 34). So, it was observed that nuclear DNA content with chromosome number had a strong positive correlation in *Curcuma* (r = 0.98) and *Kaempferia* (r = 0.96). Since, the chromosome number is not changing with the change in nuclear DNA amounts, interpretation was not possible in *Hedychium*. The Pearson's correlation coefficient for all the 9 species of Zingiberoideae resulted in r = 0.30. The weak correlation pattern is caused due to the increase in the nuclear DNA content with no change of chromosome number for *Hedychium*.

The regression analysis of the chromosome number and nuclear DNA content relationship was a perfect fit for *Curcuma* ($y = 0.016x + 1.774$, $r^2 = 0.978$) and *Kaempferia* ($y = 0.117x + 1.532$, $r^2 = 0.928$). But when the nuclear DNA content (1-C) and the chromosome number (2n) were plotted, the near zero regression coefficient was observed ($y = 18.86 + 1052$, $r^2 = 0.087$ taking 9 species into consideration. So it can be said for *Curcuma* and *Kaempferia* that chromosome number changes were due to unbiased deletion or duplication of chromosomes, as the slope of this regression approached 1.0 with increasing data. It suggests that polyploidy plays an important role ingenerating genome size variation between *Curcuma* and *Kaempferia* species. This findings is in close association of the *Eleocharis* (Zedek et al 2010). Although the result is in strong contrast with the negative correlation found for the *Cyperaceae* genus *Carex*, in which polyploidy was found to be very rare. The sudden drop of the regression coefficient (r^2) was caused by incorporation of *Hedychium* chromosome number to predict the model of change in nuclear DNA content changes are due to fission and fusion with no change in chromosome number (Fig 5e.1).

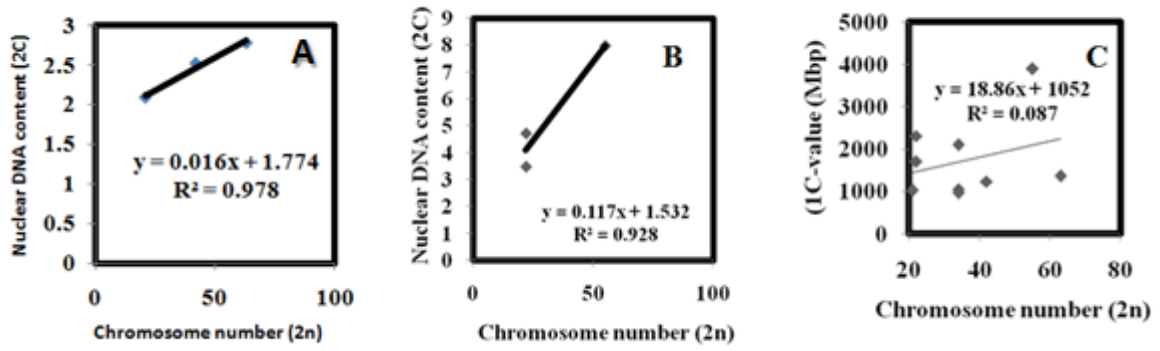

Fig 5E.1 Relationship of chromosome number and nuclear DNA content. **A.***Curcuma* **B.***Kaempferia***C.** 9 species of Zingiberoideae taken together.

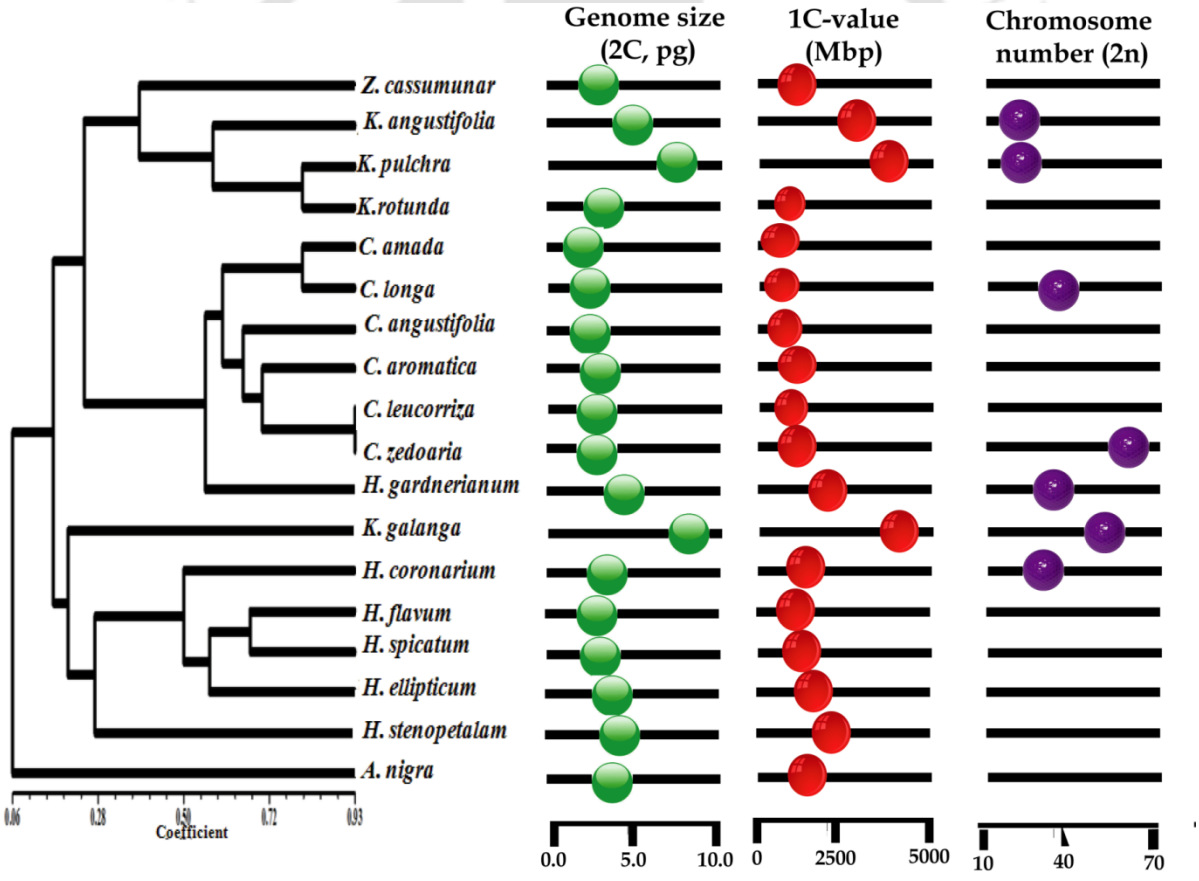

Fig 5E.2 Integration of the molecular phylogeny, cytological and flow cytometric data of Zingiberoideae

The integration of the thesis was achieved by combining the infra-generic phylogenetic tree with that of the chromosome information determined for 9 species of economic significance, nuclear DNA content data in both pg for 2C-value and Mbp for 1C-value (Fig 5E.2). In the sub family Zingiberoideae, the observed C/n-values increase toward evolutionary younger taxa and show a highly significant phylogenetic signal (Table 5E.1). The small C/n in Zingiberoideae can be explained by agmatoploidy, whereas the increase in chromosome size in more recently diverged taxa could result either from fusion or from the amplification of transposable elements, most likely LTR retro transposons.

5E.4 Conclusion

This study gives for the first time the correlated analysis of nuclear DNA content and chromosome number for the Zingiberoideae family. This concerted approach study opens the door for in depth analysis of mobile DNA elements and their distribution pattern in the genome wide

SUMMARY AND FUTURE SCOPE

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SUMMARY

Zingiberoideae is an essential group of rhizomatous medicinal and aromatic plants characterised by the presence of volatile oils and oleoresins of export value. Their usage in herbal medicine has grown significantly in recent times among health practitioners and the general public. This forces drug development and pharmaceutical companies to look forward indigenous land and knowledge for new resources, which can be used in creating and mass-marketing new drugs. However issues concerning allergic responses for usage of herbal medicine necessitate the pharmacogenomics approaches. To meet these dual objectives, current study demonstrated the ethno-pharmacological significance, molecular cytogenetics and genomics of Zingiberoideae.

The very **first phase** of the research work was undertaken to collect germplsm of the *Curcuma*, *Hedychium* and *Kaempferia* from NE parts of India. Twenty two collected species were identified with taxonomic tools and properly maintained in green house and as herbarium specimen. The ethno-botanical survey of the plants of Zingiberoideae was carried out and information was maintained for each of the plant for the future pharmacological investigations. It is the first kind of documentation of use of the Zingiberoideae species in traditional medicine. Chromosome count and their associated karyomorphological information of 9 species provided information about chromosome evolution.

Second phase involved assessing genetic diversity and relationship using molecular markers. The study revealed high amount of diversity left among the studied cultivated varieties of *Curcuma* (*C. longa*). The inter and intra-species genetic relationship for wild *Hedychium* and *Kaempferia* species of NE India was established using RAPD, ISSR and AFLP markers. The first phylogenetic map of *Hedychium* and *Kaempferia* was established which will be helpful in conservation and management. Then inter-generic phylogenetic relationship in the subfamily Zingiberoideae was studied using *Alpinia nigra* as an out group. The study revealed two clades namely the *Curcuma* clade and the *Hedychium* clade. The study also revealed the paraphyletic relationship in *Kaempferia* genus.

Third phase involved the flow cytometric estimation of nuclear DNA content of Zingiberoideae species of NE India. The flow cytometric protocol was optimised for Zingiberoideae for the first time in a new buffer system for studies on nuclear DNA content. The buffer was found suitable for a wide range of Zingiberoideae species and other species too. The variation in nuclear DNA content for 10 different tissue types of six different plants viz. *Curcuma zedoaria, C. amada, C. angustifolia, Kaempferia pulchra, Boesenbergia rotunda* and *Costus speciosus* has been established. The study revealed young leaf tissue as

the ideal material for estimation of nuclear DNA content. The nuclear DNA content among the turmeric cultivated variety (*C. longa*) across different states of NE India resulted significant differences. The study also revealed that the genetic diversity parameter (Shannon's information index) and genome size are positively correlated. The study opens the door for nuclear DNA content as the potential marker for the crop productivity. In addition, the nuclear DNA content of 26 species was estimated by flow cytometry against four different standards based on regression analysis. The fold variation in the Zingiberoideae was established.The phylogenetic tree based on nuclear DNA content grouped the Zingiberaceae species into three groups, those that show little variation; that show moderate range of variation and those that show wide range of variation. Finally the dynamics of chromosome number and nuclear DNA content was studied. The result revealed that plolyploidization played important role for generating variation in nuclear DNA content in the genera *Curcuma* and *Kaempferia*. But for the genus *Hedychium*, nuclear DNA content changes are due to fission and fusion with no change in chromosome number. Finally the integration of cytological, phylogenetic tree for inter-generic study and their respective nuclear DNA content was also enumerated.

Such an in-depth study will establish a pharmacogenomic repository for Zingiberoideae species of NE India. The identification of the species will be aided by the cytological studies molecular markers and nuclear DNA content data.

FUTURE SCOPE

Studies reported in this thesis have both theoretical and applied significance. The potential work which could be explored on the basis of present research:

- Discovery of different plant species belonging to subfamily Zingiberoideae used by different tribes and communities of NE India paves way to undertake a detailed ethobotanical study of the region.
- Chromosome analysis and engineering has been greatly advanced by fluorescence *in situ* hybridization (FISH). The technique can be applied to genome research for investigating karyotypic evolution of the studied Zingiberoideae species.
- "B Chromosomes" are the variety of supernumerary chromosomes that show conspicuous heterogeneity in their behaviour, nature and evolutionary dynamics. They are distributed widely in eukaryotes. They can vary a great deal in number both within species and between species and within populations. The intra-species and inter species differences in nuclear DNA amounts within Zingiberoideae can be elucidated.
- Mobile genetic elements or transposons are an important component of eukaryotic genomes. Among the transposable elements, retrotransposons are the largest group. They are ubiquitous in plants, though their abundance varies widely. The repetitive elements of Zingiberoideae can be characterised.
- Targeted fingerprinting marker techniques are based on the well-established practices of arbitrarily amplified DNA methods. These include transposable element based markers, conserved DNA and gene family based markers, Resistance-gene based markers, RNA-based markers, targeted fingerprinting markers etc. The genetic relationship and diversity of Zingiberoideae species can be characterised by the usage of these markers.
- Epigenetic factors such as DNA methylation and histone modifications regulate a wide range of processes in plant development. The tissue typic difference in nuclear DNA amounts of the Zingiberoideae plant can be studied on a epigenetic background.
- Flow karyotyping is a classification of mitotic metaphase chromosomes by flow cytometry according to nuclear DNA content and AT: GC ratio at high speed. Chromosome sorting is useful for genome sequencing in plants and is successful for 18 plants crop plants like barley, maize and wheat. The future study can involve sorting of chromosomes of Zingiberoideae species too.

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PUBLICATION & BIOGRAPHY

PUBLICATION

Published articles:

1. Aadi Moolam Ramesh*****, **Supriyo Basak***, Rimjhim Roy Chowdhury, Latha Rangan (2013). Flow cytometric estimation of nuclear DNA content of *Pongamia pinnata* L. ***Applied Biochemistry and Biotechnology***. DOI 10.1007/s12010-013-0553-z.
2. Rimjhim Roy Choudhury, **Supriyo Basak**, Aadi Moolam Ramesh and Latha Rangan (2013). Nuclear DNA content of Pongamia pinnata L. and genome size stability of in vitro regenerated plantlets. ***Protoplasma***. doi.10.1007/s00709-013-0544-4.
3. Tushar*, ***Supriyo Basak****, Gajen C Sarma, Rangan L. (2010). Ethnomedical uses of Zingiberaceous plants of Northeast India. Journal of Ethnopharmacology 132(1):286-96.
4. **Supriyo Basak**, Meenakshi Kumari, Amrita Kumari, Prasanna Jyothi D (2010). Production of cellulase from *Myrothecium verrucaria* NCIM 903 by solid state fermentation utilizing wheat bran as substrate. ***Dynamic Biochemistry, Process Biotechnology and Molecular Biology*** 44-45.

Manuscript under review:

1. **Supriyo Basak**, Aadi Moolam Ramesh, and Latha Rangan (2013). Molecular phylogeny of *Hedychium* from Northeast India as dissected using PCA analysis and hierarchical clustering. Plant Biosystems.
2. **Supriyo Basak** and Latha Rangan (2013) Chromosome number, Flow cytometric estimation of nuclear DNA content, phylogenetic and microscopic analysis of of Zingiberaceae of North east India. PlosOne.

Training and Workshop:

2012- Participated in 13th Indo-US workshop (IUCW), held during October 8th - 10th at IIT Guwahati, India.

2011- Participated in 13th Indo-US workshop (IUCW), held during October 14th - 16th at Manipal University, Manipal, India

2011-Hands-on Course on Flow Cytometry from Cellular and Molecular Platforms (C-CAMP) 5th-8th April, 2011.

Symposium:

S Basak and L Rangan (2012) Genome size estimation of some selected species of Zingiberaceae from North East India. 6th International symposium on the family Zingiberaceae Department of Botany, University of Calicut, Kerala- 673635, India pp-24.

Conferences:

L Rangan, **S Basak**, and AM Ramesh (2012) Ploidy Levels of Potential Biodiesel Crops Determined by Flow Cytometry. 2012 Meeting of the Society for In Vitro Biology. June 3 – 7, Bellevue, Washington.

S Basak, Tushar, V. Kesari, Archana Das, Vikash Kumar Dubey, and Latha Rangan(2010). Phylogenetic analysis in Zingiberaceae native to northeast India using RAPD markers International Conference on Genomic Sciences- Recent Trends (ICGS-2010) School of Biological Sciences, Madurai Kamraj University, Madurai-625 021. pp-120.

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SUPRIYO BASAK

Mr. Supriyo Basak joined as a doctoral student in the Department of Biotechnology, Indian Institute of Technology Guwahati (IITG), India in July 2009. He has carried out a multidisciplinary research during his doctoral study period under the joint supervision of Dr. Latha Rangan and Dr. Vikash Kumar Dubey at IITG. His research interests mainly include molecular systematics of plants.

Mr. Basak has completed B. Tech (Biotechnology) in 2005 from Bengal College of Engineering and Technology; West Bengal affiliated to West Bengal University of Technology. He passed Graduate Aptitude Test Examination (GATE) in 2007 and completed M. Tech (Biotechnology) from VIT University, Vellore in 2008. During the Masters program he worked on the utilization of agricultural wastes for production of fungal protease enzyme.

Before joining IITG for his doctoral studies, he worked at Vinayaka Mission's University's Kirupananda Variyar Engineering College, Salem, Tamil Nadu as lecturer in the Department of Biotechnology. He has carried out research work on "Production of cellulase enzyme utilizing agricultural waste by Fungi obtained from different sources", "Screening of Agricultural waste for the production of cellulase" and "Growth of *Bacillus subtilis* in liquid media containing zinc phosphide". This work got published in international journal and was presented during conferences.

Currently he has completed the doctoral study with dedication and interest in multidisciplinary research. His doctoral research mainly focused on ethno-botanical, cytogetics and flow cytometric studies on Zingiberaceae. He has undergone a hands-on Course on Flow Cytometry from Cellular and Molecular Platforms (C-CAMP) 5th-8th April, 2011. He learned the technique of DNA flow cytometry from the mentors of the workshop (Dr. Awtar Krishan) during 12th Indo-US workshop on "Applications of Flow cytometry in Plant genomics and nanotechnology" at IIT Guwahati. He has worked in the Central Image and Flow Facility of National Center for Bioloical Science, Bangalore from 24th November, 2011 to 10th December, 2011under the supervision of Dr. H. Krishnamurthy, Head of the CIFF facility to learn the techniques of flow cytometry and confocal laser scanning microscopy. During his doctoral studies he has published the work in conferences like International Conference on Genomic Sciences- Recent Trends (ICGS-2010) School of Biological Sciences, Madurai Kamraj University, Madurai, 6th International Symposium on the family Zingiberaceae. Calicut. A part of his doctoral research has been published in international peer reviewed journal and some are under review. He is keenly interested to continue further research in flow cytometric studies and dynamics of genome evolution.